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A STUDY OF HERPES SIMPLEX VIRUS
LATENCY IN CULTURED CELLS.

by

RUTH ANN ANDERSON
(née HARRIS)

A Thesis Presented for the Degree of
Doctor of Philosophy.

in

The Faculty of Science,
University of Glasgow.

Institute of Virology,
Church Street,
Glasgow G11 5JR.

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SUMMARY.

A distinctive feature of herpes simplex virus (HSV) is the ability to establish latency in the neuronal cells of the sensory ganglia. The current working model for the organization of the latent HSV type 1 (HSV-1) genome is that it persists as a circular episome associated with nucleosomes in a chromatin-like arrangement. Thus, latent HSV-1 genomes in vivo are in a physical state that differs from the characteristic unit length molecules found in virion DNA. During latency HSV-1 gene expression is restricted to a family of latency associated transcripts (LAT) detectable in the sensory ganglia of experimental animals and humans. An in vitro latency system has been utilized to investigate the molecular biology of HSV latency. The main objectives of the research were the examination of the process of reactivation, particularly the role of the HSV-1 polypeptide Vmw110 and the elucidation of the properties of mutant in1814 during latency in vitro.

The HSV-1 deletion mutant dl1403 was previously shown to be unable to reactivate latent HSV type 2 (HSV-2) in the in vitro latency system. The failure of dl1403 to reactivate latent HSV-2 suggested a role for Vmw110 in the reactivation process but did not exclude the possibility of a role for LAT or that Vmw110 acted in conjunction with other immediate early (IE) polypeptides. These aspects of reactivation were investigated using a combination of hybrid adenoviruses and inframe deletion mutants of HSV-1. Superinfection of latently infected monolayers with these viruses revealed that during reactivation in vitro there was no requirement for LAT and that the only prerequisite was a functional Vmw110. Specific features intrinsic to Vmw110 that were important for the ability of Vmw110 to activate gene expression in the absence of Vmw175 in transient transfection assays were essential for reactivation.

Mutant in1814 contains a 12 base pair (bp) inframe insertion in the gene encoding Vmw65 such that the polypeptide it encodes is unable to interact with cellular proteins and thereby transinduce IE gene expression. Southern hybridization analysis demonstrated that in1814 reactivates latent HSV-2, although less efficiently than

wild type (wt) HSV-1 or the rescuent 1814R. Infection of human foetal lung (HFL) cells at low multiplicity (0.1 particle/cell) with in1814 resulted in the efficient establishment of latency from which the majority of input particles could be recovered by superinfection with tsK at 38.5°C. By exploiting the reduction in gene expression and growth of in1814 on HFL cells, a high multiplicity latency system was developed. Importantly, mutant in1814 permitted the establishment of a latent infection directly at the physiological temperature provided the growth medium was supplemented with arabinofuranosyl cytosine (Ara-C). Infection at high multiplicity suggested that the effects of mild heat shock and the mutation were not additive, implying that they exert their effects through the same pathway involving IE gene expresssion.

The responsiveness of the latent in1814 genome to the transactivators Vmw110 and Vmw65, was determined after establishment of latency at 42°C or 37°C by superinfection of cultures with either tsK or tsK irradiated with ultraviolet light. As latency was established, the IE promoters were rapidly modified and became insensitive to transinduction by Vmw65. In analogy with the HSV-2 latency system, a functional Vmw110 supplied by the superinfecting virus was necessary for reactivation of in1814 in either the 42°C or 37°C system.

The HSV-1 terminal fragments BamHI q and s were undetectable in the nuclei of HFL cells infected with 5 particles/cell in1814 following incubation for 4 days at 42°C and 37°C in the presence of Ara-C or aphidicolin despite the detection of the HSV-1 joint spanning fragment BamHI k. Therefore, the predominant form of in1814 DNA during latency in cultured cells is non-linear. By comparison with reconstruction experiments the in1814 joint fragment was estimated to be present in quantities of 0.1-0.5 copies/cell at 37°C and 0.2-1.0 copies/cell at 42°C in a monolayer of 10⁶ cells. This is the first evidence that latent HSV-1 is "endless" in vitro and is in agreement with a previous observation that under similar conditions the latent HSV-2 genome is in a non-linear configuration.

The status of gene expression of the latent in1814 genome was examined using a variety of methodologies. In

ABBREVIATIONS.

A	adenine
ACV	acyclovir; 9-[(2-hydroxyethoxy)methyl] guanine
Ad5	adenovirus type 5
Ara-C	arabinofuranosyl cytosine
BHK	baby hamster kidney
BHV-1	bovine herpes virus type 1
bp	base pair(s)
BSA	bovine serum albumin
BVDU	(E)-5-(2-bromovinyl)-2'-deoxyuridine
BZLF1	<u>Bam</u> HI Z leftward ORF (
C	cytosine
cAMP	cyclic adenosine-5'-monophosphate
cdNA	complementary DNA
Ci	curie(s)
CNS	central nervous system
cpe	cytopathic effect
cpm	counts per minute
CRE	cAMP response element
DATD	N,N'-diallyltartardiamide
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
DEP	diethyl pyrocarbonate
dGTP	2'-deoxyguanosine-5'-triphosphate
<u>dl</u>	deletion
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside-5'-triphosphate
DTT	dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
dUTP	2'-deoxyuridine-5'-triphosphate
DW	distilled water
EBNA-1	Epstein-Barr nuclear antigen-1
EBV	Epstein-Barr virus
EDTA	sodium ethylenediamine tetra-acetic acid
EEB	electroelution buffer
G	guanine
GEB	gel elution buffer
GSCN	guanidinium thiocyanate

HCMV	human cytomegalovirus
HEL	human embryonic lung
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane
HFL	human foetal lung
HHV-6	human herpes virus type 6
HMBA	N,N-hexamethylene-bisacetamide
HSV	herpes simplex virus
HSV-1	HSV type 1
HSV-2	HSV type 2
Hu IFN- α Ly	human lymphoblastoid IFN- α
HVS	herpesvirus sa imiri
IE	immediate-early
IEC	immediate-early complex
IFN	interferon
IFN- α	human leukocyte interferon
<u>in</u>	insertion
IR _L	internal repeat long
IR _S	internal repeat short
kb	kilobase(s)
kd	kilodalton(s)
L	long
LAA	latency-associated antigen
LAT	latency-associated transcript(s)
LAT ⁺	LAT-positive
LAT ⁻	LAT-negative
MIF	macrophage inhibition factor
mLAT	minor LAT(s)
MMLV	moloney murine leukaemia virus
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
mu	map unit(s)
mwt	molecular weight
n	unspecified nucleotide
NGF	nerve growth factor
NPT	non-permissive temperature
OD	optical density
ORF	open reading frame
<u>ori</u>	origin of DNA replication
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit(s)
PNS	peripheral nervous system
polyA ⁺	polyadenylated

polyA ⁻	non-polyadenylated
PRV	pseudorabies virus
R	purine
RI	refractive index
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RS	rabbit skin
S	short
SDS	sodium dodecyl sulphate
SGB	stacking gel buffer
T	thymidine
<u>Tag</u>	<u>Thermus aquaticus</u>
TCA	trichloroacetic acid
TEMED	N,N,N,N'-tetramethylethylene diamine
TK	thymidine kinase
Tris	tris(hydroxymethyl)aminoethane
TR _L	terminal repeat long
TR _S	terminal repeat short
<u>ts</u>	temperature sensitive
U _L	unique long
U _S	unique short
UV	ultra-violet light
<u>vhs</u>	virion host shutoff
Vmw	molecular weight of viral polypeptide in kilodaltons
v/v	volume/volume
VZV	varicella zoster virus
wt	wild type
w/v	weight/volume
Y	pyrimidine

1. INTRODUCTION.

The research presented in this thesis concerns the investigation of the molecular mechanisms involved in herpes simplex virus (HSV) latency in cultured cells. The Introduction focuses on the current understanding of HSV latent infection (Section 1.3.), control of immediate early (IE) gene expression (Section 1.2.3.1.), and the properties and functions of the IE polypeptides (Section 1.2.4.). Space restrictions necessitate that cellular transformation by the human herpesviruses and lytic phase replication of HSV DNA are not discussed.

1.1. The Herpesviruses.

1.1.1. The Architectural Components of HSV Virions.

Mature HSV particles have a highly characteristic appearance. The large extracellular virions measure about 100-180nm in diameter and are composed of four major elements: the core, capsid, tegument and envelope (Wildy et al., 1960; Epstein, 1962b). The core consists of an electron dense toroidal structure, placed eccentrically within the capsid, which contains DNA that may be arranged in spools around the cylindrical mass (Epstein, 1962b; Furlong et al., 1972). The capsid measures approximately 100nm in diameter and exhibits 5:3:2 axial symmetry (Wildy et al., 1960). The capsid is composed of 150 hexameric and 12 pentameric prismatic capsomeres which are arranged in the form of an icosahedron (Wildy et al., 1960). Recent evidence suggests that all capsomeres may be composed of multiple copies of a single protein, the major capsid protein (Schrag et al., 1989). Cryo-electron microscopy and computer image analysis has provided the first three dimensional structure of full and empty capsids of herpes simplex virus type 1 (HSV-1) (Schrag et al., 1989). In the study by Schrag et al. (1989) the nucleocapsid was determined to be organized into three distinct layers: i) an outer shell of capsomeres, ii) an intermediate proteinaceous layer in T=4 icosahedral lattice and iii) an inner layer

containing the HSV genome. The tegument is defined as the structure located between the capsid and the envelope. In thin section, the tegument appears as an amorphous matrix (Epstein, 1962b; Roizman and Furlong, 1974). The amount of tegument material is variable even within the same infected cell (Fong et al., 1973) and is genetically determined by the virus (McCombs et al., 1971). The outermost structure of the herpes virion is the envelope, which is probably derived from the host nuclear membrane (Morgan et al., 1959; Epstein, 1962a). The envelope is a triple layered structure covered with periodic projections composed of HSV glycoprotein molecules (Morgan et al., 1959; Wildy et al., 1960; Epstein, 1962b; Stannard et al., 1987).

The family Herpesviridae has been classified into three subfamilies, namely Alpha, Beta and Gammaherpesvirinae, by the Herpes Study Group appointed by the International Committee on Taxonomy of Viruses, based on the criteria of host range, length of reproductive cycle, cytopathology and characteristics of latent infection (Roizman et al., 1981). The characteristics of the human herpes viruses and the subfamilies assigned to them are summarized in Table 1.1.

1.1.2. Human Herpesviruses and Disease.

In humans, infection with HSV-1 typically occurs early in life. The disease is often inapparent and frequently undifferentiated from other childhood illnesses. Despite recovery from the primary oropharyngeal infection, the virus establishes a long term latent infection during which clinically overt symptoms are not apparent. The latent infection serves as a reservoir for periodic recurrent attacks of herpes labialis. In addition, HSV-1 recurrences involving the epithelium of the cornea (herpes keratitis) may have severe sequelae including blindness. Herpes genitalis is usually a sexually transmitted disease seen mainly in adults, although herpes simplex virus type 2 (HSV-2) infection can also occur at birth (Whitley et al., 1985). Most genital herpes infections are caused by HSV-2 but a minority can be attributed to HSV-1. Like HSV-1, HSV-2 establishes a latent infection in the sensory ganglia with

Table 1.1. CLASSIFICATION OF HUMAN HERPESVIRUSES ACCORDING TO THEIR BIOLOGICAL PROPERTIES, AND SUBDIVISION INTO SUBFAMILIES.

Alphaherpesvirinae.

Variable host range in tissue culture.

Short reproductive cycle.

Cytolytic infection.

Latent infection established primarily in ganglia.

EXAMPLES	Herpes Simplex Virus 1	(Human Herpes Virus 1)
	Herpes Simplex Virus 2	(Human Herpes Virus 2)
	Varicella Zoster Virus	(Human Herpes Virus 3)

Betaherpesvirinae.

Restricted host range.

Relatively short reproductive cycle; slow progressing infection in cell culture.

Cytomegalic infection.

EXAMPLES	Human Cytomegalovirus	(Human Herpes Virus 5)
	Human Herpes Virus 6	

Gammaherpesvirinae.

Host range limited to family or order of natural host.

In vitro replication in lymphoblastoid cells; some also cause lytic infections in some types of epitheloid and fibroblastoid cells.

Latent virus is frequently demonstrated in lymphoid tissue.

EXAMPLE	Epstein-Barr Virus.	(Human Herpes Virus 4)
---------	---------------------	------------------------

occasional recurrence of disease at the periphery.

The pathogenesis of varicella (chicken pox) involves a primary subclinical infection of the oropharynx or conjunctiva, followed by viraemia and the development of generalized systemic epithelial eruptions (Weller, 1983; Gelb, 1985). Herpes zoster (shingles) is the consequence of endogenous reactivation of latent varicella zoster virus (VZV) from sensory ganglia (Vafai et al., 1988) and it is thought that immunosuppression combined with increasing age predisposes individuals to recurrences (Schimpff et al., 1972). In rare cases, both HSV and VZV may spread to the central nervous system (CNS) during the primary or possibly recurrent infection causing encephalitis and meningitis (Kennedy, 1987). The fatalities are high and many survivors suffer permanent neurological damage.

Productive Epstein barr virus (EBV) infections in humans are often asymptomatic but clinically apparent infection is manifest as infectious mononucleosis, typically amongst adolescents. EBV is also associated with the development of Burkitts lymphoma and nasopharyngeal carcinoma (Neiderman et al., 1976; Miller, 1985).

Transplacental human cytomegalovirus (HCMV) infection is a major viral cause of congenital abnormalities in newborns (Alford and Britt, 1984). However, most HCMV infections acquired after birth are benign and subclinical, and when apparent a syndrome resembling EBV mononucleosis with atypical lymphocytes is observed. HCMV infection has been reported in immunocompromised patients, particularly recipients of organ transplants (Ho, 1982) and AIDS sufferers.

Salahuddin et al. (1986) described the isolation of a novel herpesvirus, human herpes virus type 6 (HHV-6), from the lymphocytes of patients with AIDS and lymphoproliferative disorders. Recent surveys indicate that 80-90% of humans develop antibodies to HHV-6 early in life (Saxinger et al., 1988; Okuno et al., 1989) and there is evidence to suggest that HHV-6 is the causative agent of childhood exanthum subitum (roseola infantum) (Yamanishi et al., 1988). It is unclear whether this virus is responsible for any other human disease(s).

1.1.3. The Structure of the Human Herpesvirus Genomes.

Herpesviruses may be defined on the basis of their nucleic acid characteristics (Roizman, 1982), and are diverse in terms of their biological properties (Section 1.1.2.) and the size, structure and base composition of their genomes (Honess, 1984). The structures of the six human herpesvirus genomes are illustrated in Figure 1.1. The base composition of herpes virus DNA varies from relatively low % G+C, eg. VZV has a G+C content of 46% (Ludwig et al., 1972; Davison and Scott, 1986), to classified viruses with a mean G+C% composition $\geq 60\%$, eg. HSV-1, HSV-2 and EBV strain B95-8 have G+C contents of 68.3%, 69%, and 59.9% respectively (McGeoch et al., 1988; Kieff et al., 1971; Baer et al., 1984). Betaherpesviruses occupy the middle of the range of observed compositions, eg. HCMV has a G+C content of 57-58% (Crawford and Lee, 1964; Plummer et al., 1969). The complete nucleotide sequence is available for HSV-1 (McGeoch et al., 1985, 1986, 1988; Perry and McGeoch, 1988), EBV (Baer et al., 1984) and VZV (Davison and Scott, 1986). Chee et al. (1990) determined the sequence of HCMV strain AD169 and presented an analysis of the potential protein coding content of the genome. For HSV-2 strain HG52 most of the unique short (U_S) region has been sequenced (McGeoch et al., 1987). Recently, approximately 22 kilobases (kb) of sequence from the genome of HHV-6 strain U1102 was obtained (Lawrence et al., 1990) that included sequences previously recognized as having significant homology with a region of the HCMV genome (Efsthathiou et al., 1988).

The HSV genome is a linear duplex molecule of approximately 100×10^3 kilodaltons (kd) in the native state (Becker et al., 1968; Kieff et al., 1971). Electron microscopic studies showed that the HSV-1 genome is a complex molecule divided into two covalently linked regions long (L) and short (S). Both the L and S components consist of unique sequences, unique long (U_L) and U_S , flanked by large terminal repeats [terminal repeat long (TR_L) and terminal repeat short (TR_S)] (Sheldrick and Berthelot, 1974; Figure 1.1.). The position that separates the L and S

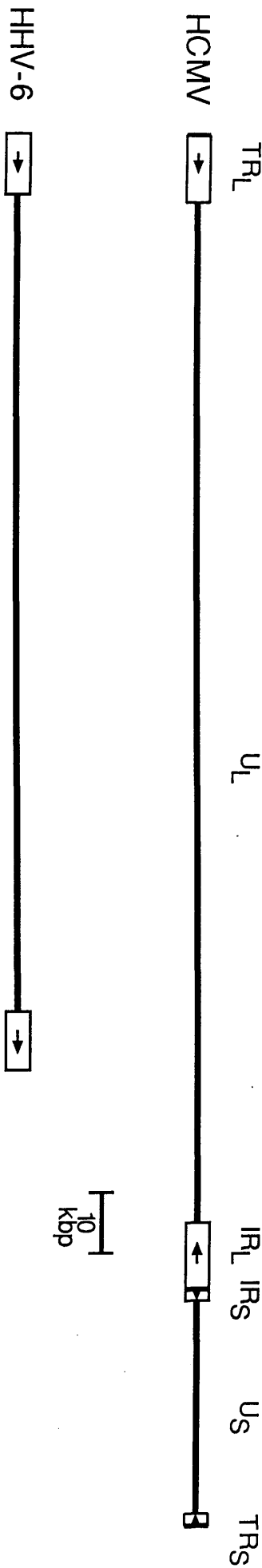
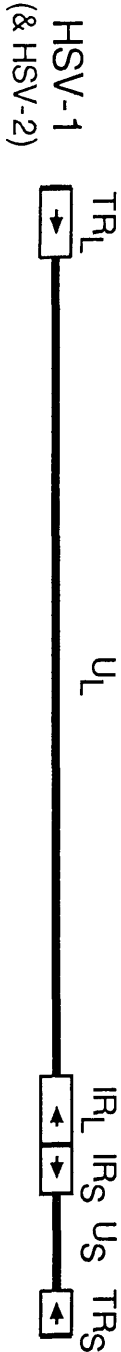
Figure 1.1. GROSS STRUCTURES OF THE HUMAN HERPESVIRUS GENOMES.

The genome structures of the six known human herpesviruses are presented as linear molecules. Bold lines represent unique sequences and repeat elements are shown as open boxes with arrows indicating the relative sequence orientation.

Abbreviations used in the diagram :

U _L	unique long
U _S	unique short
I/TR _L	internal/terminal repeat long
I/TR _S	internal/terminal repeat short
D _L	direct repeat left
D _R	direct repeat right
MIR	major internal repeat
ori	origin of DNA replication

This figure was reproduced from McGeoch (1989).



segments is termed the joint [internal repeat long (IR_L), internal repeat short (IR_S)] and is identical to but inverted with respect to the terminal repeats (Sheldrick and Berthelot, 1974). The redundant repeat regions bracketing the L and S segments were designated ab,b'a' and a'c',ca respectively (Wadsworth et al., 1975; Figure 1.2.). The repeats are not identical except that they share an ^{approximately} 400 base pair (bp) a sequence that is defined as the minimum terminal repetitive sequence required for optimal circularization (Wadsworth et al., 1976). The a sequence can vary in length and it has been reported that many junctions between L and S contain only a single a sequence (Wagner and Summers, 1978). HSV DNA extracted from plaque purified virions is heterogeneous and consists of equimolar amounts of four isomeric molecules differing from each other in the relative orientation of the U_L and U_S segments with respect to the joint (Sheldrick and Berthelot, 1974; Hayward et al., 1975; Delius and Clements 1976; Wilkie and Cortini 1976; Clements et al., 1976; Roizman, 1979). Amongst this population of molecules a prototype arrangement was chosen for mapping purposes (Roizman, 1979; ^{McGeoch et al. 1988} Figure 1.3.). The internal recombination events that generate all four isomers are directed by the a sequence (Mocarski and Roizman, 1982). There is evidence to suggest that each isomer is able to take part in the production of infectious progeny (Davison and Wilkie, 1983a) and participate in a latent infection (Efsthathiou et al., 1986). HSV-1 and HSV-2 have closely related genome sequences that are essentially colinear (Davison and Wilkie, 1983b) and share a high degree of homology (70-80%) in coding sequences (McGeoch et al., 1987). The greatest differences occur in the major repeat elements and in U_S (Davison and Wilkie, 1981; McGeoch et al., 1987).

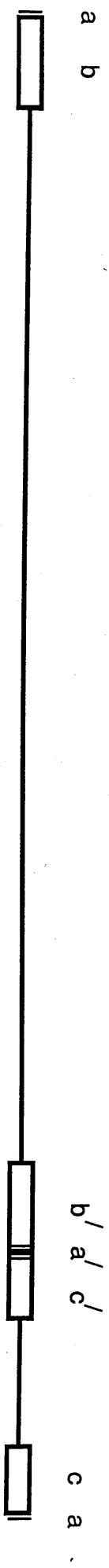
1.2. Lytic Infection.

1.2.1. Initial Cell-Virus Interactions.

The earliest steps in HSV replication are viral attachment and penetration. The initial step in the

**Figure 1.2. THE POSITIONS OF THE a, b AND c SEQUENCES IN THE
REPEAT REGIONS OF THE HSV GENOME.**

The locations of the a, b and c sequences in the HSV genome are indicated. The a', b' and c' sequences are identical to a, b and c in inverted orientation. The copy number of a sequences present at the junction between the L and S components of the genome is variable but often equal to one.



infection of cells by both HSV serotypes is thought to be the binding of virus particles to a heparan sulphate moiety on the surface of the cell (WuDunn and Spear, 1989). Subsequent additional interactions are required between other cell receptors and the virus envelope for complete adsorption (Addison et al., 1984). Penetration into the target cell has been observed by fusion and endocytosis (Smith and De Harren, 1974), although the principle mode of entry is via a membrane fusion route (Morgan et al., 1968; Para et al., 1980; Johnson et al., 1984). It was shown that mild acidic conditions can reversibly inhibit penetration of HSV and that the extent of pH dependence was determined by the HSV strain (Rosenthal et al., 1989). The adsorption and penetration of HSV are probably mediated by multiple interactions between envelope glycoproteins encoded by the viral genome and cell surface components. Genetic evidence has demonstrated that the glycoproteins designated gB, gD and gH are essential for virus infectivity and entry into the cell (Little et al., 1981; Ligas and Johnson, 1988; Cai et al., 1988; Desai et al., 1988). Neutralizing monoclonal antibodies specific for each of these glycoproteins blocked viral penetration with minimal effect on virion adsorption (Fuller and Spear, 1987; Highlander et al., 1987, 1988; Fuller et al., 1989). An in vitro binding assay has indicated that gC may be involved in complex formation with cellular surface structures (Kuhn et al., 1990).

1.2.2. Alteration of Host Macromolecule Synthesis.

The inhibition of host macromolecule synthesis is a multistage process (Fenwick, 1984). A primary phase of host shutoff of protein synthesis is mediated by one or more components of the virion (Fenwick and Walker, 1978; Fenwick et al., 1979; Hill et al., 1985), designated virion host shutoff (vhs), that is accompanied by degradation of cellular mRNAs (Fenwick and McMenamin, 1984; Schek and Bachenheimer, 1985). A late (secondary) shutoff function reduces the remaining levels of host protein synthesis and requires expression of viral early (β) and late (γ) genes (Fenwick and Clark, 1982; Read and Frenkel, 1983). Several

viable vhs mutants have been isolated that were defective in the inhibition of host protein synthesis (Read and Frenkel, 1983; Kwong and Frenkel, 1987). Mapping of the vhs mutation in HSV-1 (KOS) to position 0.6 map units (mu) on the genome suggested that the virion-mediated shutoff function was encoded by U_L41 (Kwong et al., 1988). Inactivation of the vhs gene is associated with increased stability of viral transcripts (Read and Frenkel, 1983; Kwong and Frenkel, 1987) and recent studies indicate that mutations in vhs lengthen the cytoplasmic half-life of many HSV-1 mRNAs representing all kinetic classes (Oroskar and Read, 1989). Therefore, the vhs protein may play a role, in conjunction with transcriptional controls, in determining the abundance and kinetics of expression of several viral mRNAs.

The vhs product has not been characterized, but it is postulated that the vhs protein may be a virus-encoded RNase or may function by activating a pre-existing cellular enzyme (Oroskar and Read, 1989). The degree of virion-mediated suppression of host protein synthesis varies amongst different strains of HSV. A hybrid virus, 17G41, derived from HSV-1 strain 17⁺ (weak vhs activity) with the HSV-2 strain G (strong vhs activity) U_L41 homologue recombined into the thymidine kinase (TK) gene was used to determine if instability of IE mRNA in the presence of cycloheximide was co-inherited with strong host shutoff function (Fenwick and Everett, 1990a). In the diploid 17G41 recombinant the HSV-2 (G) characteristic dominated and controlled both functions. However, in a mixed infection the weak shutoff function of HSV-1 (17⁺) was dominant. To account for these observations, Fenwick and Everett (1990a) hypothesised that in the infected cell U_L41 must activate a cellular nuclease and function as a dimeric molecule.

The protein product of gene U_L41 does not have an essential secondary role as a structural component of the virion, since deletion mutants within this gene can be isolated (Fenwick and Everett, 1990b).

In contrast to HSV induced host shutoff, the synthesis of some cellular proteins is stimulated during HSV infection (LaThangue et al., 1984; Macnab et al., 1985; Kemp et al., 1986; Patel et al., 1986). Recent results have indicated

that a HSV-1 IE protein (Vmw63) is obligately required for the accumulation of a 40kd cellular protein, in combination with an additional factor present in infected cells (Estridge et al., 1989). Overexpression of an abnormal form of Vmw175 is responsible for induction of cellular stress proteins (Notarianni and Preston, 1982; Russell et al., 1987). Human fibroblast cell lines infected at the non-permissive temperature (NPT) with a temperature sensitive (ts) mutant defective for virus entry induced the expression of a 56kd cellular protein (Preston, 1990).

1.2.3. Transcription of HSV Genes.

The transcription of viral DNA occurs in the cell nucleus and is directed by an unmodified host RNA polymerase II (Ben-Zeev and Becker, 1977; Costanzo et al., 1977). Viral mRNAs are processed in a similar manner to cellular messages by 5' capping, 3' polyadenylation and internal methylation (Bachenheimer and Roizman, 1976; Silverstein et al., 1973, 1976). The sequence YGTGTTY (where Y is a pyrimidine) located 30bp downstream of the polyadenylation signal AATAAA is thought to be involved in the efficient processing of HSV transcripts (McLauchlan et al., 1985). Only a small proportion of HSV mRNAs are derived by splicing, notably those of IE-1 (Perry et al., 1986), IE-4/5 (Watson et al., 1981; Rixon and Clements, 1982), U_L15 (Costa et al., 1985; McGeoch et al., 1988) and the latency associated transcripts (LAT) (Wagner et al., 1988b; Weschler et al., 1988a). Colinear mRNA families from several regions of the HSV-1 genome that possess individual promoters and 5' termini but which share 3' terminal sequences have been described (McLauchlan and Clements, 1983; Watson et al., 1983; Rixon and McGeoch, 1984; Draper et al., 1986).

Productive infection of cells with HSV involves the coordinated temporal control of viral transcripts that is ordered into three phases referred to as IE (α), β and γ (Swanstrom and Wagner, 1974; Jones and Roizman, 1979; Clements et al., 1977). During the replicative cycle five IE genes are initially transcribed in the absence of de novo protein synthesis (Kozak and Roizman, 1974; Clements et al.,

1977). Expression of early genes is dependent on the synthesis of functional IE proteins (Honess and Roizman, 1974; Preston, 1979b; Everett, 1984a; O'Hare and Hayward, 1985a). Maximum expression of late (γ_1) and "true" late (γ_2) genes requires the activities of IE and early gene products as well as viral DNA synthesis (Honess and Roizman, 1974). However, transcription of γ_1 genes can be detected at moderately high levels before the onset of DNA replication whereas γ_2 gene expression is strictly dependent upon DNA synthesis (Holland et al., 1980; Johnson and Everett, 1986a). McLauchlan et al. (1989) demonstrated the presence of a virus-induced heat labile factor in infected cells, which selectively increased cleavage at a late HSV polyadenylation site, that functioned in an in vitro cleavage assay and in the context of the viral genome. Polyadenylation site selection may be involved in the regulation of HSV gene expression, although examination of polyadenylation sequences employed at IE, early and late times does not suggest a correlation between stimulation of processing activity and temporal class (McLauchlan et al., 1989).

On the basis of open reading frame (ORF) predictions the HSV-1 genome was estimated to encode 70 unique polypeptides (McGeoch et al., 1988; Figure 1.3.). The properties of the five IE proteins induced by HSV infection are described in detail in Section 1.2.4. Many early genes have been identified as coding for proteins that are essential for viral origin-dependent DNA synthesis and enzymes involved in nucleotide metabolism that are largely non-essential (TK, ribonuclease reductase, dUTPase, uracil-DNA glycosylase and alkaline exonuclease; Table 1.2.). Late genes consist of those that encode structural proteins and proteins involved in virion assembly (Table 1.2.)

1.2.3.1. Regulation of HSV IE Genes.

Biochemical transformation of cells with a TK gene controlled by the IE-3 promoter revealed that enhancement of IE-TK gene expression required a factor associated with a superinfecting TK⁻ virus (Post et al., 1981). Mutant tsB7

Figure 1.3. ORGANIZATION OF THE GENES ENCODED BY HSV-1.

The prototype arrangement of the HSV-1 genome is illustrated over four consecutive lines relative to the indicated scales (upper and lower scales are map units and kilobases respectively). The locations of predicted ORFs are shown as arrows and the proposed polyadenylation sites of each transcript are indicated with a vertical bar. The positions of the DNA origins of replication are represented as an X (Stow, 1982; Spaete and Frenkel, 1982; Stow and McMonagle, 1983; Weller et al., 1985). Many HSV-1 ORFs have been assigned functions and are listed in Table 1.2.

This figure was reproduced from McGeoch et al. (1988).

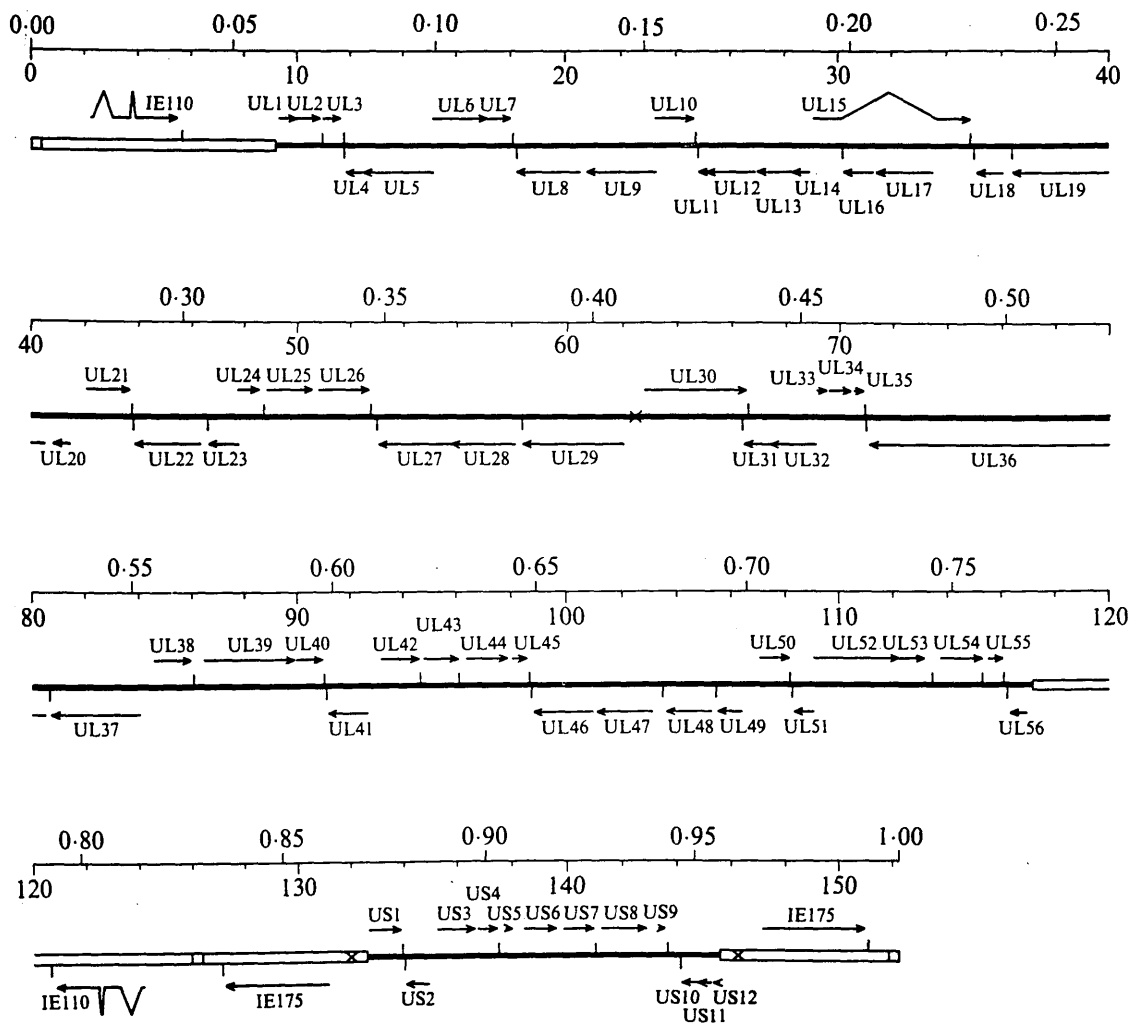


Table 1.2. PROPERTIES OF HSV-1-ENCODED PROTEINS.

GENE	FUNCTION OR PROPERTIES	ADDITIONAL KEY REFERENCES
IE110:	IE transcriptional regulator	
U _L 2	: uracil-DNA glycosylase	Mullaney <u>et al.</u> , 1990
U _L 5	: DNA helicase-primase component	Crute <u>et al.</u> , 1989
U _L 6	: virion protein	
U _L 8	: DNA-helicase-primase component	Crute <u>et al.</u> , 1989
U _L 9	: DNA replication origin binding protein	Olivo <u>et al.</u> , 1988
U _L 10	: possible membrane insertion protein	
U _L 12	: deoxyribonuclease	
U _L 13	: protein kinase	Smith and Smith, 1989
U _L 18	: capsid protein	Rixon <u>et al.</u> , 1990
U _L 19	: major capsid protein	
U _L 20	: possible membrane insertion protein	
U _L 22	: virion glycoprotein H	
U _L 23	: thymidine kinase	
U _L 25	: probable virion structural protein	
U _L 26	: DNA packaging protein	Rixon <u>et al.</u> , 1988
U _L 27	: virion glycoprotein B	
U _L 28	: capsid protein	Addison <u>et al.</u> , 1990
U _L 29	: single stranded DNA binding protein required for replication	
U _L 30	: replicative DNA polymerase	
U _L 34	: virion protein	
U _L 36	: very large tegument protein	
U _L 37	: DNA binding protein, possible role in packaging	Shelton <u>et al.</u> , 1990
U _L 38	: capsid protein essential for	Pertuiset <u>et</u>

assembly	<u>al.</u> , 1989; Rixon <u>et al.</u> , 1990
U _L 39 : large subunit of ribonucleotide reductase	
U _L 40 : small subunit of ribonucleotide reductase	
U _L 41 : virion host shutoff function	Kwong <u>et al.</u> , 1988
U _L 42 : ds DNA binding protein, subunit of DNA polymerase that increases the efficiency of polymerization	Gottlieb <u>et al.</u> , 1990
U _L 44 : virion glycoprotein C	
U _L 47 : tegument protein	McLean <u>et al.</u> , 1990
U _L 48 : tegument protein, activator of IE gene expression	
U _L 50 : deoxyuridine triphosphatase	
U _L 52 : helicase-primase component	Crute <u>et al.</u> , 1990
U _L 53 : possible membrane protein	
U _L 54 : Vmw63, IE transcriptional regulator	
IE175: Vmw175, IE transcriptional regulator	
U _S 1 : Vmw68, IE protein	
U _S 3 : protein kinase	
U _S 4 : virion glycoprotein G	
U _S 5 : putative glycoprotein	
U _S 6 : virion glycoprotein D	
U _S 7 : virion glycoprotein I	
U _S 8 : virion glycoprotein E	
U _S 9 : tegument phosphoprotein	
U _S 10 : virion protein	
U _S 12 : Vmw12, IE protein	

Many HSV-1 ORFs have been assigned functions and are listed above. References are cited in the original paper (McGeoch et al., 1988) and where appropriate additional references are quoted to update the table. Unmentioned ORFs have no assigned property/function.

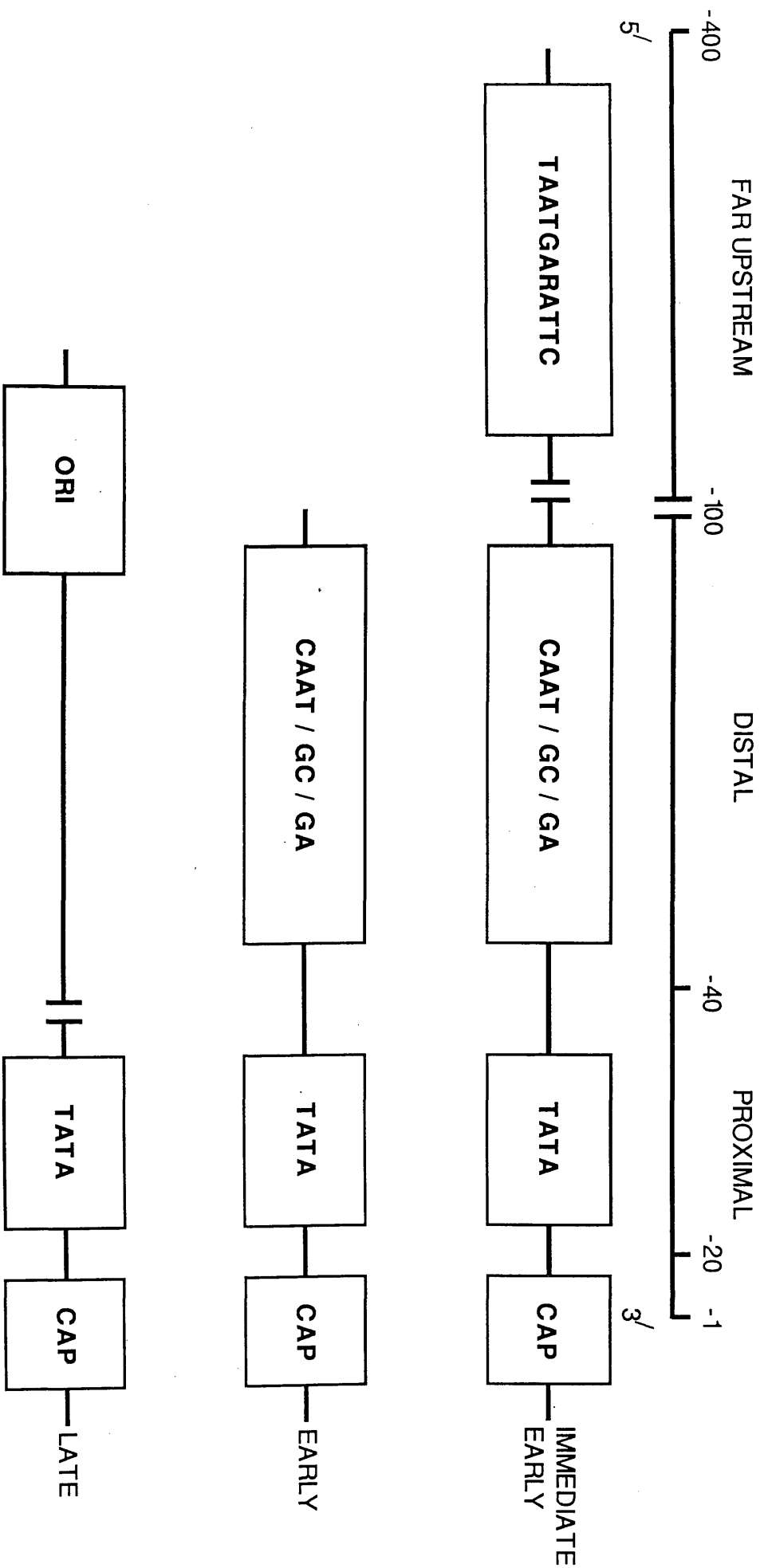
that is defective for capsid uncoating could stimulate IE gene expression at the NPT indicating that the component mediating IE induction was located external to the nucleocapsid (Knipe et al., 1981; Batterson and Roizman, 1983). The identity of the transinducing factor was determined by cotransfection assays using HSV restriction fragments and chimeric constructs in which TK gene expression was directed by an IE-specific promoter (Campbell et al., 1984). TK activity was stimulated by a 2.7×10^3 bp subclone of the BamHI f fragment (Campbell et al., 1984). This region was shown by analysis of HSV-1 x HSV-2 recombinants to encode a major virion phosphoprotein present in the tegument at approximately 400-600 molecules per particle (Marsden et al., 1978; Heine et al., 1974). Sequence data indicated the presence of a single ORF (U_L48) in BamHI f predicted to encode a polypeptide of molecular weight (mwt) 50,342 (Dalrymple et al., 1985). Hybrid-arrested translation and immunoprecipitation studies demonstrated that U_L48 encoded a 65kd protein termed Vmw65 (Campbell et al., 1984).

The signals responsible for the regulation of IE genes were first identified using a recombinant virus in which the TK early promoter was replaced with the 5' upstream sequences of an IE gene (Post et al., 1981). In cells infected with the recombinant virus TK was transcribed and processed under IE conditions (Post et al., 1981). Examination of sequence data and deletion analysis of the far upstream region of HSV IE genes revealed a conserved cis-acting homology, the TAATGARAT motif (where R represents a purine), distinct from the promoter and TATA box elements, that was essential for IE gene expression in response to Vmw65 (Mackem and Roizman, 1982a,b,c; Cordingley et al., 1983; Kristie and Roizman, 1984; Lang et al., 1984; Preston et al., 1984; Gaffney et al., 1985; Bzik and Preston 1986). DNA sequences flanking the TAATGARAT motif contribute to the positive stimulatory effect of Vmw65 (Kirstie and Roizman, 1984; Preston et al., 1984; Bzik and Preston, 1986; Triezenberg et al., 1988a; Figure 1.4.). The GA-rich sequences are found in close proximity to at least one TAATGARAT consensus (Kristie and Roizman, 1984) and increase

Figure 1.4. DNA SEQUENCES REQUIRED FOR EFFICIENT REGULATION OF HSV-1 GENES.

The control regions of IE genes contain a far-upstream consensus TATAGARATTC necessary for stimulation of IE gene expression by Vmw65. Distal elements found upstream of IE and early promoters include a CAAT box homology, and GC- or GA-rich motifs. Late promoters consist of a TATA box, capsite region and an active origin of replication.

This figure was reproduced from Johnson and Everett (1986a).



the TAATGARAT mediated responsiveness to Vmw65 (Bzik and Preston, 1986; Triezenberg et al., 1988a). The regulatory region of Vmw175 contains several copies of a GC-rich tract which is the binding site for the eukaryotic transcription factor Sp1 (Jones and Tjian, 1985). In addition to these regulatory elements, the upstream region between IE-3 and IE4/5 contains an enhancer-like sequence (Lang et al., 1984; Preston and Tannahill, 1984). In the absence of Vmw65, the enhancer can stimulate expression from fused genes in an orientation independent manner and at a considerable distance from a heterologous promoter (Lang et al., 1984; Preston and Tannahill, 1984). However, unlike prototype enhancers, this HSV enhancer is not functional when inserted downstream of a gene (Preston and Tannahill, 1984).

While the function of Vmw65 correlates with its ability to interact with cis-acting target sequences, Vmw65 does not itself have any intrinsic DNA binding properties either for DNA containing the TAATGARAT motif or non-specifically for double stranded DNA (Marsden et al., 1987; Preston et al., 1988). A combination of DNaseI footprinting and gel retardation assays indicated that a physical association of Vmw65 with cellular components was required for specific binding to TAATGARAT elements (McKnight et al., 1987; O'Hare and Goding, 1988; Preston et al., 1988; Gerster and Roeder, 1988). Gel retardation assays demonstrated the presence of cellular factors in uninfected HeLa cell nuclear extracts which had the capacity to interact with a short DNA fragment containing the TAATGARAT element, that resulted in the formation of a specific complex (Preston et al., 1988; O'Hare and Goding, 1988). Importantly, analysis of infected cell nuclear extracts detected the presence of a novel complex with very low electrophoretic mobility termed an immediate early complex (IEC) (Preston et al., 1988). Addition of a NP40 treated virion preparation to uninfected nuclear extracts also directed the formation of a DNA-protein complex that was indistinguishable from the IEC (Preston et al., 1988). Therefore, IEC formation is dependent upon both cellular and viral factors.

Evidence that Vmw65 is a component of IEC is derived from several experiments: i) incorporation of a monoclonal

antibody specific for Vmw65 in gel retardation assays further decreased the migration of the IEC complex (Preston et al., 1988) and ii) addition of affinity purified or in vitro translated Vmw65 to uninfected cell extracts induced complex formation (McKnight et al., 1987; Preston et al., 1988; O'Hare and Goding, 1988).

The identity of cellular factors involved in the co-operation with Vmw65 in IEC formation was suggested by comparison of the TAATGARAT motif with the sequence elements known to be recognized by other transcription factors. The TAATGARAT consensus is homologous to the octamer binding site for a cellular protein, nuclear factor III, located in the adenovirus DNA replication origin (Prujijn et al., 1986). Also, the nuclear factor III binding sequence is similar to the conserved octamer motif, ATGCAAATNA, found in the promoter or enhancer regions of many cellular genes (Falkner and Zachau, 1984). Transcription factors that interact with this motif have been identified and purified. One such factor, called OTF-1, is an ubiquitous protein present in a wide range of cell types. Initial evidence concerning the nature of the cellular factors interacting with Vmw65 was suggested when short oligonucleotides containing the octamer binding motif formed a complex with and competed efficiently for proteins binding to the TAATGARAT element (O'Hare and Goding, 1988). Gel retardation experiments using a DNA fragment derived from the far-upstream region of the Vmw110 promoter and purified OTF-1 in the absence of other proteins detected a complex previously found in uninfected nuclear extracts. Addition of a Vmw65 in vitro translation product to the reaction mix induced inefficient IEC formation when highly purified OTF-1 was used (Gerster and Roeder, 1988). The level of IEC was significantly increased by the further addition of an OTF-1 depleted HeLa cell extract in reconstitution experiments (Gerster and Roeder, 1988). In summary, the cumulative evidence demonstrates that IEC formation requires the recognition of cis-acting sequences that contain the TAATGARAT motif by Vmw65 with OTF-1 and at least one other cellular factor.

Inspection of the predicted amino acid sequence of Vmw65 revealed a region at the carboxy terminus which contained a high frequency of acidic residues (Dalrymple et al., 1985) that resembled the highly negatively charged regions described in the transcriptional activation domains of the prototype yeast regulatory proteins GCN4 (Hope and Struhl, 1986) and GAL4 (Gill and Ptashne, 1987). Independent analyses of Vmw65 deletion (dl) mutants have determined the requirements for transactivation of IE gene expression in transient expression systems (Triezenberg et al., 1988b; Greaves and O'Hare, 1989). Deletion of the acidic domain located within the carboxy terminal 78 amino acids virtually abolished the trans-acting function of Vmw65 (Triezenberg et al., 1988b; Greaves and O'Hare, 1989). This same domain activates gene expression when fused to a heterologous DNA binding domain from GAL4, demonstrating that this region functions independently of the remaining Vmw65 molecule (Sadowski et al., 1988). This chimeric activator protein selectively inhibited activated transcription but not basal transcription in an in vitro assay (Berger et al., 1990) thought to be by sequestration of a cellular adaptor molecule responsible for positioning the activation domain in contact with the basic transcriptional machinery (Ptashne, 1988).

Deletion of the carboxy terminal domain does not have a significant effect on IEC formation. Instead, deletion of N-terminal amino acids or insertion mutagenesis of the same region disrupts the capacity of Vmw65 to interact with OTF-1 and other cellular factors (Ace et al., 1988; Triezenberg et al., 1988b; Greaves and O'Hare, 1989). A stably transformed cell line that constitutively expressed a truncated Vmw65 lacking the activation domain was impaired in the ability to support a productive HSV-1 infection particularly at low multiplicity of infection (moi) (Friedman et al., 1988). This implied that the amino terminal portion of the polypeptide sequestered cellular DNA binding proteins rendering them unavailable for IEC formation with Vmw65. Recently, Greaves and O'Hare (1990) finely mapped two regions in the primary sequence of Vmw65 which were necessary for protein-protein interactions in vitro.

Deletion of residues between amino acids 49-75 and 380-388 abolished the transactivating function of Vmw65 suggesting that at least two domains are required for efficient complex assembly which may act independently or act as a single domain formed by juxtaposition of these regions in the folded protein (Greaves and O'Hare, 1990).

In conclusion, the results described above demonstrate that the functional domains of Vmw65 are distinct. The acidic domain is dispensable for tertiary complex formation but is absolutely necessary for the transcriptional activating function of Vmw65. In contrast, amino terminal residues are critical for complex formation.

Analysis of defined insertion (in) mutants of Vmw65, generated by inframe insertion of a short oligonucleotide, in transient transfection assays demonstrated that the functional domains necessary for stimulation of IE gene expression and structurally important regions were separable (Ace et al., 1988). Characterization of the mutant plasmids revealed that the ability to form an IEC correlated with the ability to transinduce expression of a heterologous gene. Marker rescue experiments using the mutated constructs and ts2203, which has a mutation in the HSV-2 homologue of Vmw65 such that assembly of virus particles is affected, determined whether a given 12bp insertion had affected a domain crucial for the structural integrity of Vmw65. The protein synthesised by the plasmid with an insertion at HaeIII site 14 (in14) was unable to transactivate IE gene expression but rescued ts2203, implying that a viable virus containing the in14 insertion could be constructed. The resulting HSV-1 mutant virus in1814 has demonstrated the importance of Vmw65 in a lytic infection (Ace et al., 1989). Mutant in1814 produces a defective Vmw65 that is unable to interact with cellular proteins and stimulate IE gene expression, resulting in an up to 10-fold decrease in the amount of IE proteins (Ace et al., 1989). The reduction in growth and cell type dependency of in1814 is most dramatic on human foetal lung (HFL) cells, on which the particle:plaque forming units (pfu) ratio is typically between 1×10^4 - 5×10^4 compared to values of 10-50 for wild type (wt) HSV-1 or the rescued virus 1814R (Ace et al.,

1989). Preinfection of monolayers with ultra-violet light (UV) inactivated wt HSV-1 or prior transfection of a plasmid expressing the HSV-1 transactivator Vmw110 complements the plaquing efficiency of in1814 close to that of HSV-1 or 1814R (Ace et al., 1989). Thus, the reduced growth of in1814 is a consequence of an inability to initiate plaque formation, particularly at low moi, rather than structurally defective particles.

The results presented by Ace et al. (1989) suggested that transinduction by Vmw65 is not essential for HSV IE gene expression at high moi and that the effect of the viable mutation in Vmw65 could be overcome by increased gene dosage of IE promoters. Consistent with this hypothesis is the recent report that the amount and the rate of in1814 production is increased following infection of a cell line stably expressing Vmw65 compared to control Vmw65-negative cells (Werstuck et al., 1990).

Mutant in1814 has been employed to investigate the role of Vmw65 in latent infections (Steiner et al., 1990; Section 1.3.5.).

1.2.3.2. Regulation of Early Gene Expression.

Early gene promoters respond to the IE transactivators Vmw175 and Vmw110 (Everett, 1984a; Gelman and Silverstein, 1985; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a). Initial experimentation aimed at delineating the sequence requirements of early promoters employed deletion mutagenesis of the upstream region of the TK gene and microinjection of Xenopus oocytes (McKnight et al., 1981; McKnight and Kingsbury, 1982). Three separate components necessary for accurate and efficient transcription initiation in vivo were identified in the 5' flanking DNA of the TK gene that consisted of a derivative of the conserved TATA and CAAT box homologies and two GC-rich elements which bind the cellular transcription factor Sp1 (McKnight et al., 1981; McKnight and Kingsbury, 1982; Jones et al., 1985; Figure 1.4.). Analysis of mutated, transfected TK genes activated by superinfection with HSV indicated that the requirements for regulation of TK are similar to those for

TK gene expression in basal expression systems, implying that IE gene products did not bind directly to specific sequence elements within the TK promoter (Eisenberg et al., 1985; El Karez et al., 1985). Importantly, the same domains identified by transient transfection or microinjection of Xenopus oocytes were shown to be crucial in TK mRNA expression in the context of the viral genome (Coen et al., 1986). The signals required for regulation of another early promoter, gD, were determined by short term transfection assay and found to lie within 83bp upstream of the RNA capsite (Everett, 1983). Like the TK control region, the gD promoter contained several functionally important domains including a TATA box homology and two upstream GA-rich elements (Everett, 1984b). Additional evidence that HSV early promoters lack virus inducible sequences was derived from the observation that IE proteins activated the expression of a hybrid cellular β -globin gene when fused to the gD promoter (Everett, 1983, 1984b, 1985) and that a HSV-1 recombinant encoding the hybrid β -globin gene expressed β -globin mRNA as a HSV early gene product (Smiley et al., 1987).

1.2.3.3. Regulation of Late Gene Expression.

The sequence requirements for regulated expression of U_S11, which encodes a 21kd protein regulated with the kinetics of a "true" late gene, have been described (Johnson et al., 1986; Figure 1.4.). Sequences between -31 and +39, which include a consensus TATA box homology at position -26, were sufficient for expression of U_S11 from an origin (ori) positive template (Johnson and Everett, 1986b). Distal regions analogous to those found upstream of the TK and gD promoters (Everett, 1984b; Eisenberg et al., 1985) were absent from the U_S11 promoter (Johnson and Everett, 1986b). A similar sequence requirement for regulation of gC has been demonstrated (Homa et al., 1986). It is accepted that viral DNA synthesis is essential for achieving abundant late gene expression. A >95% reduction in U_S11-initiated transcription was observed from replication inhibited genomes and ori-negative plasmids (Johnson and Everett,

1986a; Johnson et al., 1986). Despite these data, HSV-1 prototype late gene promoters may be activated in the absence of DNA replication (DeLuca and Schaffer, 1985; Godowski and Knipe, 1985; Johnson et al., 1986). Although U_S11 gene expression was reduced 50-100 fold in the presence of phosphonoacetic acid, low levels of U_S11 mRNA remained detectable using a sensitive assay technique (Johnson et al., 1986). Similarly, under conditions of DNA synthesis inhibition, the family of gC mRNAs accumulated to 10% of the level detected in cells infected by a replicating wt virus (Godowski and Knipe, 1985). DeLuca and Schaffer (1985) demonstrated that the late L42 promoter identified by Hall et al. (1982) was inducible by superinfection with wt virus or ts Vmw175 mutants when fused to an indicator gene on an ori-negative plasmid. It was postulated that a dependence on increased copy number of intrinsically weak promoters and/or altered template structure as a consequence of DNA replication may explain why efficient late gene expression requires DNA synthesis (Johnson and Everett, 1986a). Prior IE and early gene expression is indirectly needed for activation of late gene transcription. In this regard, analysis of HSV mutants indicated that the IE polypeptides Vmw63 and Vmw68 are important for expression of some late genes (Sacks et al., 1985; Sears et al., 1985b).

1.2.4. Properties and Functions of HSV IE Polypeptides.

In HSV-1 infected cells the five IE genes encode the following polypeptides: Vmw175, Vmw110, Vmw63, Vmw68 and Vmw12 (Honess and Roizman, 1974; Watson and Clements, 1978; Figure 1.3.). At least three of these IE gene products are involved in transcriptional regulation of later classes of viral genes (Preston, 1979a; Everett, 1984a, 1986; Sacks et al., 1985; Stow and Stow, 1986) and most IE proteins have some affinity for DNA in vitro (Hay and Hay, 1980).

The IE-3 gene is diploid as it is transcribed entirely within the repeats bounding U_S (Rixon et al., 1982). The gene product, Vmw175, is a large nuclear phosphoprotein (Pereira et al., 1977) which is essential for lytic replication of HSV-1, since analysis of mutants with ts

lesions in this gene demonstrated that a functional Vmw175 is continuously required for production of early and late mRNAs (Preston, 1979a; Watson and Clements, 1980). Fine structure mapping indicated that the defect in tsK was caused by a single missense amino acid substitution in the central region of the polypeptide at residue 475 (Davison et al., 1984) which resulted in the synthesis of an abnormal polypeptide at the NPT that accumulated in the cytoplasm and was not post-transcriptionally modified to forms of lower electrophoretic mobility nor poly(ADP-ribosyl)ated (Preston, 1979b; Preston and Notarianni, 1983). Other mutants, tsD and tsT, with lesions located towards the carboxy terminal end of Vmw175 have been described (Preston, 1981). The characterization of mutants tsK, tsD and tsT identified separate features of Vmw175 later demonstrated to be important for the activity of Vmw175 in vitro and in vivo (DeLuca and Schaffer, 1987,1988; Paterson and Everett, 1988a,b; Paterson et al., 1990).

Vmw175 shares sequence homology with a VZV protein with predicted mw of 140kd (Davison and Wilkie, 1983b; McGeoch et al., 1986) that is a potent activator of gene expression in transient transfection assays (Everett, 1984a; Everett and Dunlop, 1984; Inchauspe et al., 1989). A recombinant Vmw175-negative virus expressing the VZV 140kd protein under HSV-1 IE-3 control was able to grow on normal cell lines in the absence of Vmw175, implying that the heterologous proteins are functionally related (Disney and Everett, 1990). However, complementation was not absolute as the hybrid virus, HSV-140, exhibited a multiplicity-dependent gene expression defect that was independent of cell type (Disney and Everett, 1990).

DNA binding immunoassays indicated that Vmw175 can associate specifically with nucleotide sequences present in HSV-1 DNA (Faber and Wilcox, 1986). The conserved core sequence 5'-ATCGTC-3' for Vmw175 binding is present in promoter regions of IE-1, IE-3 and gD (Faber and Wilcox, 1986; Kristie and Roizman, 1986a,b; Muller, 1987). Beard et al. (1986) using a modified in vitro transcription assay demonstrated that the initiation of gD mRNA synthesis is stimulated about 5-fold by the addition of a partially

purified preparation of Vmw175. Vmw175 binding sequences have been identified in the gD gene 5' to the start site for gD mRNA synthesis and within the gD coding region (Tedder et al., 1989). It has been reported that there is a direct correlation between the number of consensus binding sites on the DNA template and the degree of stimulation of gD transcripts initiated in vitro (Tedder et al., 1989). In contrast, a recent report demonstrated that interactions between Vmw175 and binding sequences in the TK promoter contributed little to the transactivation of TK gene expression in vivo (Imbalzano et al., 1990). Many promoters activated by Vmw175 do not contain the consensus sequence (Everett, 1988b; McGeoch et al., 1985,1988). Indeed, Vmw175 can bind to alternative nucleotide sequences unrelated to the core consensus in the promoter regions and 5' transcribed non-coding regions of some early and late genes (Michael et al., 1988; Tedder et al., 1989; Imbalzano et al., 1990). Therefore, the sequence of the binding site may reflect properties of a cellular transcription factor that is involved in interactions with Vmw175 (Everett, 1987b) or alternatively, the core sequence may represent only one of a family of diverse binding sites recognised by Vmw175. Gel retardation assays using infected cell extracts and probes spanning -4 to +27 relative to the 5' terminus of IE-3 mRNA detected binding of Vmw175 to its own transcription start site (Muller, 1987). The target sequence included the consensus binding site 5'-ATCGTC-3' which has been implicated in an autoregulatory role in the context of the viral genome (DeLuca and Schaffer, 1988).

Recent mutagenesis studies of the gene encoding Vmw175 have revealed regions of functional importance (DeLuca and Schaffer, 1987, 1988; Paterson and Everett, 1988a,b). Paterson and Everett (1988a) described the construction of a large panel of inframe insertion and deletion mutations in the coding region of IE-3. The activities of the mutant IE-3 polypeptides were assessed using short term transfection assays and defined two regions conserved between Vmw175 and the VZV 140kd protein that were important for the transactivation of a HSV-1 early gene and repression of its own promoter (Paterson and Everett, 1988a). The same study

identified a nuclear localization signal between amino acids 682-774 (Paterson and Everett, 1988a). A subsequent study using nuclear extracts from HeLa cells determined that there was a general correlation between the ability to transactivate/repress gene expression in transfection assays and impaired ability to bind the IE-3 cap site in gel retardation assays (Paterson and Everett, 1988b). The integrity of an amino acid region located at 315-384 was essential for transcriptional control and DNA binding by Vmw175 (Paterson and Everett, 1988a,b). Independent analyses using a less refined methodology defined similar functionally important domains (DeLuca and Schaffer, 1987,1988). DeLuca and Schaffer (1987) reported that the carboxy terminal 40% and the first 90 amino acids of Vmw175 were not crucial for regulation of transcription in transient transfection assays. A viral mutant expressing a truncated polypeptide, encoding the first 590 residues of Vmw175, was nonetheless able to form a novel DNA complex under conditions of elevated salt concentration (DeLuca and Schaffer, 1988).

Characterization of mutants with ts lesions in Vmw175 has revealed that the mode of action of the polypeptide is complicated further since mutants unable to bind DNA in vitro at the NPT possess the capacity to stimulate early gene expression albeit at reduced levels (DeLuca et al., 1984; Paterson et al., 1990). Mutant ts1225 produced a polypeptide defective for autoregulation of IE-3 gene expression whilst retaining the ability to bind to the IE-3 cap site that is important for autoregulation during virus infection (DeLuca and Schaffer, 1988).

The gene encoding IE-1 is present in the HSV-1 genome in two copies as it is located entirely within the long repeats (Preston et al., 1978) and transcripts arising from this gene are spliced (Perry et al., 1986). The protein product, Vmw110, is a nuclear phosphorylated protein capable of binding to DNA in crude nuclear extracts (Periera et al., 1977; Hay and Hay, 1980; Ackerman et al., 1984). Vmw110 has been shown to stimulate a wide range of promoters including those from all classes of HSV genes (O'Hare and Hayward, 1985a,b; Gelman and Silverstein, 1985,1986,1987; Quinlan and

Knipe, 1985; Everett, 1986; Mavromara-Nazos et al., 1986; Shapira et al., 1987; Sekulovich et al., 1988) and heterologous promoters such as the SV40 early promoter (O'Hare et al., 1986; Everett, 1988b) and the HIV LTR (Mosca et al., 1987). Introduction of Vmw175 and Vmw110 by cotransfection on separate plasmids into the same cell results in the synergistic stimulation of gene expression (Everett, 1984a, 1986). Short term transfection assays have defined at least five mutation-sensitive regions that contribute to the function of Vmw110 (Everett, 1987a, 1988a). Mutations in the carboxy region of the protein resulted in a significant reduction in the expression of a heterologous gene in the presence of Vmw175 (Everett, 1987a). A region of the IE-1 polypeptide between amino acids 130-160 contains three partially overlapping copies of a cysteine-rich motif that has been implicated in the binding of metal ions and DNA (Berg, 1986). Inframe deletion mutants in which this cysteine-rich domain in the second exon was removed retained some transactivating function in the presence of Vmw175 (Perry et al., 1986; Everett, 1987a). However, this region was absolutely essential for the activity of Vmw110 in the absence of Vmw175 (Everett, 1988a). The functional significance of the potential metal ion binding finger is unknown since Vmw110 in nuclear extracts is retained on metal chelate affinity columns although the interaction is not dependent on the zinc finger (R.D. Everett, unpublished observations). It is clear, however, that this domain is crucial for the intrinsic ability of Vmw110 to stimulate gene expression. Immunofluorescence studies determined that amino acids 474-509 contained highly basic residues that were important but not completely sufficient for nuclear localization (Everett, 1988a).

Mutant viruses with ts lesions in the IE-1 gene have not been reported but large deletion mutants with the IE-1 coding sequences removed in both copies have been described (Stow and Stow, 1986; Sacks and Schaffer, 1987). The mutant viruses were able to grow on normal cell lines although the yield of virus was 10- to 100-fold lower than that obtained with wt virus (Stow and Stow, 1986; Sacks and Schaffer,

1987). Mutant dl1403 has a 2kb deletion in both TR_L and IR_L such that the majority of the sequences coding for Vmw110 and about 750bp of the 3' region of LAT are removed (Stow and Stow, 1986). The growth deficiency of dl1403 is manifest only at low moi since baby hamster kidney (BHK) cells infected with 5pfu/cell of dl1403 or wt HSV-1 revealed no significant difference in the levels of polypeptide synthesis and DNA replication or encapsidation (Stow and Stow, 1986). Analysis of a number of mutant viruses with small inframe deletions in both copies of the IE-1 gene, particularly mutants FXE and D22, revealed a correlation between the importance of regions defined by transfection experiments and those required for transactivation of gene expression and growth at low moi (Everett, 1989). These data suggest that Vmw110 is not essential for efficient plaque formation at high moi but its presence confers a selective growth advantage at low moi.

The product of the IE-2 gene, Vmw63, is a nuclear phosphoprotein (Perreira et al., 1977; Ackerman et al., 1984). Initial evidence that IE-2 performed an essential role in gene regulation was derived from the biochemical characterization of four ts mutants with mutations residing in the gene encoding Vmw63 (Sacks et al., 1985). Phenotypically, the expression of some HSV genes were affected, in particular certain IE polypeptides were overproduced and late protein synthesis was significantly reduced (Sacks et al., 1985). Inclusion of a plasmid-borne copy of Vmw63 in cotransfection assays in combination with Vmw110 and Vmw175 resulted in an increased stimulation of at least one late gene coding for the major capsid protein (Everett, 1986). Similarly, efficient expression of other HSV late gene products, gB and gC, were dependent on a functional Vmw63 (Rice and Knipe, 1988; Rice et al., 1989). It has been demonstrated that Vmw63 can negatively affect the expression of several HSV-1 IE, early and late promoters in transfection studies (Sekulovich et al., 1988; Su and Knipe, 1989). Functional activities of Vmw63 investigated using a set of carboxy truncated IE-2 polypeptides has demonstrated that the regions important for repression and transactivation are separable (Rice et al., 1989). The

domain required for the negative regulatory activity of Vmw63 was located between amino acids 263-406 whilst the carboxy terminal residues were necessary for the transactivating function (Rice et al., 1989). Likewise, an independent report using inframe insertion and deletion mutants throughout the Vmw63 gene demonstrated that the ability to repress or activate target gene expression were distinct (Hardwicke et al., 1989). Additional mutagenic analysis of the IE-2 polypeptide in which Vmw63 variants (Rice et al., 1989) were recombined into the viral genome in place of the wt gene identified two distinct transactivating domains responsible for the stimulation of γ_1 genes in combination with viral DNA replication and expression of γ_2 genes (Rice and Knipe, 1990).

Deletion of a large portion of the carboxy end of the IE-4 gene resulted in a mutant virus with apparently normal growth properties implying that Vmw68 is not an essential polypeptide (Post and Roizman, 1981). Subsequent studies revealed that on some cell lines growth was restricted and that production of a late chimeric γ_2 -TK protein was decreased (Sears et al., 1985b). This disparity can be explained if the Vmw68 function can be replaced by a cell factor produced in certain but not other cell lines.

The function of Vmw12 is unclear. In contrast to other IE proteins, Vmw12 is located in the cytoplasm of the infected cell and is not phosphorylated (Periera et al., 1977; Hay and Hay, 1980; Marsden et al., 1982). On the basis of these characteristics it is likely that Vmw12 has a unique role amongst the IE polypeptides that does not involve regulation of transcription. Mutant viruses deleted for Vmw12 (or the HSV-2 equivalent) are viable, indicating that the product of the IE-5 gene is not essential for growth in tissue culture (Umene, 1986; Longnecker and Roizman, 1986; Brown and Harland, 1987).

1.2.5. Cleavage and Encapsidation of Viral DNA.

The process of cleavage and packaging of newly synthesised concatemeric viral DNA into preformed empty capsids is a coupled process (Ladin et al., 1982; Deiss and

Frenkel, 1986; Deiss et al., 1986). Cleavage of concatemeric molecules occurs within the a sequence situated between adjacent viral genomes (Davison and Wilkie, 1981; Mocarski and Roizman, 1982) and appears to be accompanied by amplification of an a sequence. The a sequence consists of unique and directly repeated elements and in HSV-1(F) has the structure DR1-Ub(DR2)₂₂-(DR4)³-Uc-DR1 (Mocarski and Roizman, 1982). The precise cleavage/packaging signal was mapped within the conserved unique regions in a using an amplicon transfection-propagation assay (Deiss and Frenkel, 1986; Deiss et al., 1986). Virus stocks derived from transfections with constructs deleted in the Uc element lacked the ability to propagate defective genomes whilst deletions in Ub affected efficiency of amplicon seed replication (Deiss et al., 1986). Deiss et al. (1986) described a model for cleavage and encapsidation of viral DNA wherein the juxtaposition of two directly repeated junctions resulted in a amplification according to the double stranded break and gap repair mechanism proposed by Szostak et al. (1983). The cleavage/encapsidation process may be complicated further by the presence of a "head full" recognition element that selects the juxtaposed a sequence once an upper limit of DNA has been packaged (Frenkel et al., 1976). Fragments shorter than full-length HSV molecules can become encapsidated but maturation is incomplete and the capsid remains unenveloped (Vlazny et al., 1982). HSV-1 mutants ts1201 and ts1203 are defective in encapsidation of viral DNA at the NPT but can be distinguished by the inability of ts1201 to process the Vmw40 family of polypeptides at the NPT (Preston et al., 1983; Matz et al., 1983). Rixon et al. (1988) utilized ts1201 and ts1203 to determine that Vmw40 was transiently associated with empty capsids and Vmw40 removal was coupled with packaging of viral DNA into capsids. Two distinct DNA-protein complexes have been found in infected cell nuclei that consisted of HSV specific proteins bound to probes containing components of the a sequence (Chou and Roizman, 1989). Interestingly, one complex contained the virally encoded DNase and the other complex consisted of equimolar concentrations of a structural protein (U_L36) and an

unidentified 140kd polypeptide (Chou and Roizman, 1989). Recent characterization of an alkaline exonuclease null mutant also suggested that this protein was involved in the processing of viral DNA into capsids. The mutant was competent for growth and DNA synthesis on normal cells, although efficient production of infectious virus particles required a wt nuclease activity supplied in a transformed cell line (Weller et al., 1990). The protein product of gene U_L37 has been identified as a γ_1 non-structural, single stranded DNA binding protein containing a potential ATP-binding domain in the amino acid sequence, that has been postulated to function at late times during DNA replication in the cleavage/packaging process (Shelton et al., 1990).

1.3. Latent Infection.

Latency is central to the natural history of HSV-associated disease. Latent infections occur between episodes of acute disease and involves the persistence of the viral genome in specific tissues without evidence of replication or clinical disease whilst retaining the capacity to reactivate and yield infectious progeny. Although many questions remain unanswered, the 20 years following the report by Stevens and Cook (1971) has seen the knowledge of HSV latency expand. This section aims to highlight important aspects of HSV latency research, beginning with the development of animal models and tissue culture latency systems that, in combination, continue to be vital for studies on HSV latency. The role of viral gene expression during the stages of latency, and the state of the latent DNA will be discussed in detail. The involvement of host determined factors in establishment, maintenance and reactivation of HSV latency will also be considered.

1.3.1. Models Employed in the Study of HSV Latency.

1.3.1.1. Animal Models of HSV Latent Infection.

Several animal model systems have been developed for the study of HSV latency in vivo. The most commonly used

laboratory animals include mice, rabbits and guinea pigs. The favoured route of inoculation is at a peripheral site such as the eye (Knotts et al., 1973; Walz et al., 1974; Nesburn et al., 1977;), lip (Walz et al., 1974; Sekizawa et al., 1980), ear pinna (Hill et al., 1975), footpad (Stevens and Cook, 1971, 1973a,b; Scriba, 1975; Scriba and Tatzber, 1981) and vagina (Scriba, 1976; Stanberry et al., 1985). Some models utilized alternative routes of inoculation including intraperitoneal (Richards et al., 1981), intracerebral (Hill et al., 1975; Watson et al., 1980) and haemotogenous (Cook and Stevens, 1976). Following peripheral inoculation in experimental animals the virus moves centripetally to the sensory nerves and corresponding ganglia and then to the CNS.

The first animal system described in which latency was established in a predictable site was the mouse footpad model (Stevens and Cook, 1971,1973a,b; Walz et al., 1974). Infection of the rear footpad with HSV-1 or HSV-2 resulted in a local cutaneous infection that was followed by an acute infection of the sciatic nerve, sacrosciatic spinal ganglia, dorsal roots and brain. During the productive infection, infectious virus could be readily isolated from each of the above listed tissues. Virus could be isolated from the spinal ganglia after 48hr, titres peaked by day 4 and declined thereafter to undetectable levels by day 7. Animals that recovered from acute phase infection and posterior paralysis within 3 weeks harboured latent virus in the ipsilateral spinal ganglia. Latent virus could be rescued up to 3 months later by cocultivation on rabbit skin (RS) cells but not by direct assay of homogenized tissue (Stevens and Cook, 1971).

A latent infection can be established in the cervical dorsal root ganglia subsequent to intradermal inoculation of the mouse ear pinna (Hill et al., 1975). Erythema developed within 2-5 days post inoculation and was cleared by day 21, although episodes of erythema recurred sporadically over a period of several months. A variety of stimuli such as UV irradiation and stripping of the ear with cellophane tape, increased the frequency of recurrent herpetic disease (Hill et al., 1978; Harbour et al., 1983). The reproducibility of

the model described by Hill and co-workers is dependent on mouse strain. In general, the incidence of virus isolated from the site of infection following stripping is greater in outbred strains compared to inbred strains exemplified by BALB/c (Harbour et al., 1981). The mouse ear model has been valuable in the study of factors that influence recurrent disease.

Intraocular inoculation and scarification of the mouse cornea induced an acute ocular infection followed by the establishment of a latent infection in the trigeminal ganglia (Knotts et al., 1974; Walz et al., 1974). Spontaneous shedding of virus in eye secretions occurred rarely in mice recovered from the primary infection (Tullo et al., 1982). However, a recent report described the development of a model in which virus shedding and recurrent herpetic disease occurred in the mouse eye (Shimeld et al., 1990b). Recurrent eye disease was only observed in mice with eyes that remained undamaged after the initial infection and virus shedding was efficiently induced by a combination of UV light and immunosuppressive drugs (Shimeld et al., 1990b). To date the mouse eye model has been useful for the study of viral factors involved in latency. The model described by Shimeld et al. (1990b) should prove useful in the study of recurrent herpetic keratitis.

Reactivation of HSV to produce an acute infection can be induced in latently infected mice by neurectomy (Walz et al., 1974; Price and Schmitz, 1978), application of chemicals to the skin (Hill et al., 1978; Harbour et al., 1983; Hill et al., 1983b), physical stimuli like UV irradiation (Blyth et al., 1976; Harbour et al., 1983), or stripping the ear with cellophane tape (Hill et al., 1978; Blyth et al., 1980a; Harbour et al., 1981, 1983) and by intratracheal injection of pneumonococci or mucin (Stevens et al., 1975).

The rabbit eye model has been used for the study of recurrent disease (Stevens et al., 1972; Knotts et al., 1973; Nesburn et al., 1977). After corneal inoculation latency was established in the trigeminal ganglia. Ocular shedding post day 20 was considered to be spontaneous reactivation and occurred frequently (Berman and Hill,

1985). Iontophoresis of 0.01% epinephrine at 0.8mA for 8min on 3 consecutive days induced ocular reactivation (Hill et al., 1985, 1986). The advantage of the rabbit eye model is the ability to study induced or naturally occurring reactivation events.

The guinea pig model for HSV infection shares many features with genital herpes infections in humans, including a natural route of inoculation, a self-limiting initial infection characterized by vesiculo-ulcerative lesions, and neurological and urological symptoms (Stanberry, 1986). Intravaginal infection of guinea pigs with HSV-2 caused inflammation of the external genital tract and extensive formation of local lesions resembling primary genital herpes in humans (Scriba, 1976; Stanberry, 1985). A latent infection ensued in the lumbrosacral dorsal root ganglia with frequently occurring mild lesions (Scriba, 1976; Scriba and Tatzber, 1981; Stanberry et al., 1985). The frequency and duration of recurrent episodes declined significantly with time (Scriba, 1976; Stanberry et al., 1985).

The drawbacks of animal models for studying HSV latency are their complexity and expensiveness, and that none exactly mimic the human situation in all respects.

1.3.1.2. Tissue Culture Latency Sytems.

Tissue culture, or in vitro, latency sytems inherently lack the complexities of whole animal model systems; specifically serum antibody, cell mediated immunity, hormones and interferon (IFN), which are known to modulate virus replication, are not present. Although this can be levied as a criticism of tissue culture latency systems, it is these same characteristics that make such systems an attractive option for studying HSV latency, enabling the investigator to ask simple biochemical questions with reproducible results. It has been proposed that the ideal in vitro latency system should possess the following features: i) a host cell derived from neuronal tissue, ii) subsistence of infected cells, iii) persistence of the entire viral genome without production of infectious virus particles and iv) maintenance of the viral genome in a state

that can be stimulated to produce infectious progeny (Levine et al., 1980). Additionally, in the light of recent developments, it should also be required that the latent genome is "endless" and viral gene expression be restricted to the production of LAT during the latent phase of infection. In reality no in vitro latency system that fulfils all these criteria has yet been described.

Several cell systems have been utilized where a persistent or latent HSV infection has been achieved due to the intrinsic properties of the cell type or by artificial manipulation of the environmental conditions surrounding the cell. In vitro latency systems have employed transformed cells of neuronal origin (Lancz et al., 1976; Vahlne and Lycke, 1977, 1978; Adler et al., 1978; Doller et al., 1979; Levine et al., 1980; Nilheden et al., 1985a,b), primary rodent neuronal cells (Zeigler and Herman, 1980; Wigdahl et al., 1983; Wilcox and Johnson, 1987,1988; Wilcox et al., 1990), primary human neurones in culture (Wigdahl et al., 1984b) and cells of non-neuronal origin (O'Neill et al., 1972; O'Neill, 1977; Colberg-Poley et al., 1979a,b; Wigdahl et al., 1981,1982b; Nishiyama and Rapp, 1981; Youssoufian et al., 1982).

The replication of HSV-1 strain KOS has been shown to be inhibited in chemically transformed and differentiated cells derived from rat neuronal tissue (Adler et al., 1978; Levine et al., 1980). Reduced growth of HSV-1 (KOS) on this cell line is dependent on multiplicity and temperature of infection. At 39°C and low multiplicity, virus DNA synthesis was blocked although synthesis of some viral macromolecules occurred, specifically DNA polymerase, thymidine kinase and some structural proteins (Adler et al., 1978). The production of virus could not be stimulated by temperature reduction to 37°C or 34°C or by treatment of neuroma cultures with UV light (Adler et al., 1978; Levine et al., 1980).

The growth of HSV on a mouse neuroblastoma cell line, clone C1300, was restricted compared to other murine cell types (Vahlne and Lycke, 1977,1978). Kemp and Latchman (1989) have recently shown that this effect was due to the failure of IE gene expression. Sodium butyrate pretreatment

of C1300 cells reversed HSV restriction by increasing production of IE polypeptides and IE mRNA species (Ash, 1986; Kemp and Latchman, 1989). At very low moi a persistently infected culture was produced that rarely released infectious virus particles (Vahlne and Lycke, 1977). The production of virus progeny required that each C1300 cell be infected with greater than one virus particle (Vahlne et al., 1981). Hyperresistant derivatives of the C1300 cell line (RII cells) have been generated by successive exposure of survivors of HSV infection to increasing virus dose (Nilheden et al., 1985a). A non-replicating state could be established in a hyperresistant culture after infection with 0.1 pfu/cell HSV-1 and incubation of the monolayer in the presence of neutralizing antibody for two passages. Thereafter, in the absence of antibody, superinfection of RII cells with HSV-2 resulted in the production of HSV-1. The amount of quiescent HSV-1 recovered in this manner decreased with increasing passage number, which suggested that the HSV genomes did not replicate concomittantly with the cellular DNA (Vahlne et al., 1985b).

Wilcox and Johnson (1987) have described an important in vitro latency system that is based upon the cultivation of primary sympathetic neurones isolated from the cervical ganglia of neonatal rats. Treatment of these cultures with 20 μ M fluorodeoxyuridine ensured that approximately 95% of cells were neuronal, the remainder being schwann and fibroblast cells. Infection of cells with 0.5 pfu/cell of HSV-1 strain F followed by the addition of 2% anti-HSV human serum enabled the virus to coexist in the host cell for up to 5 weeks without obvious cytotoxicity and detectable viral antigen expression. The efficiency of this system was increased by incubaton of latently infected cultures in the presence of 50 μ M acyclovir (ACV) (Wilcox and Johnson, 1988). Supplementation of the culture media with guinea pig anti-mouse nerve growth factor (NGF) antibodies induced the production of virus progeny in a maximum of 53% of monolayers depending on the initial moi, which implied that maintenance of a stable latent infection in this system required NGF (Wilcox and Johnson, 1987,1989). Transient

fluctuations in the concentration of NGF were sufficient to induce reactivation (Wilcox et al., 1990). Wilcox and co-workers (1990) employed monoclonal antibodies directed against the NGF receptor to demonstrate that it is the specific binding of NGF to its receptor that is crucial for the induction of reactivation. Like NGF deprivation, short term inhibition of protein synthesis stimulated reactivation of virus from rat sympathetic or sensory neurones. It was postulated that this occurred because the cycloheximide block prevented the synthesis of an unstable host factor that is an inhibitor of reactivation and controlled by NGF (Wilcox et al., 1990). The production of LAT as well as a more controversial latency-associated antigen (LAA) has been detected in this system (Doerig et al., 1990, abstracts of the 15th International Herpesvirus Workshop, Washington DC, USA).

There is evidence to suggest that peripheral non-neuronal sites may support a latent infection in vivo, which adds credence to the use of more accessible non-neuronal cell types in the study of HSV latency in vitro (Scriba, 1981; Hill et al., 1983a; Cook et al., 1983; Al-Saadi et al., 1988; Clements and Subak-Sharpe, 1988; Clements and Jamieson, 1989; Claoué et al., 1990).

The report by O'Neill et al. (1972) set a precedent for the use of chemical manipulation of cultured cells to establish a latent infection and consequently was emulated and developed further (Rapp, 1984). O'Neill et al. (1972) described a system in which a HSV-2 lytic infection was obviated by pretreatment of human embryonic lung (HEL) cells with an inhibitor of viral and cellular DNA synthesis. By 4 days post inoculation infectious virus was undetectable and remained so for at least 5-6 days after arabinofuranosyl cytosine (Ara-C) withdrawal. The time between the disappearance of infectious virus and its reappearance defined the latent period. During this period O'Neill et al. (1972) determined that the proportion of infectious centres in HSV-2 latently infected HEL cells was very low. On average only 1 in 4000 cells retained the capacity to produce infectious particles. Prolonged treatment of latently infected cultures with Ara-C did not ultimately

prevent the subsequent development of cytopathic effect (cpe) after removal of Ara-C. Temperature elevation from 37°C to 39.5°C-40°C immediately after removal of Ara-C was employed as a modification to extend the period of latency up to 128 days in HEL cells (O'Neill, 1977). Reactivation of latent HSV-2 could be stimulated by temperature downshift to 37°C (O'Neill, 1977; Colberg-Poley et al., 1979a,b) or by superinfection with either wt HCMV (Colberg-Poley et al., 1979b) or by superinfection with HCMV ts mutants representing four different complementation groups unable to synthesise DNA at the NPT (Colberg-Poley et al., 1981). Scheck et al. (1987), using HSV-1 ts mutants blocked at various stages in the virus lytic cycle, demonstrated that a (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) sensitive viral gene product(s) expressed by the superinfecting virus genome was required for induction of reactivation.

In analogy with the HSV-2 system described above, Wigdahl et al. (1981) reported an in vitro model reliant upon Ara-C and increased incubation temperature to establish a latent HSV-1 infection. Superinfection of latently infected HEL cultures with HCMV or HSV-2 ts mutants activated virus replication which was characterized as parental input HSV-1 by plaque reduction assay in the presence of BVDU, restriction endonuclease digest analysis of EcoRI, HindIII, and XbaI cleavage patterns and titration at the NPT and permissive temperature (Wigdahl et al., 1981, 1982a). Wigdahl et al. (1982b) successfully increased the sensitivity of the existing system by treatment of cells with a combination of the antiviral compounds BVDU and human leukocyte interferon (IFN- α) prior to infection with HSV-1. In this manner, infection with 2.5 pfu/cell HSV-1 resulted in a significant increase in the number of infectious centres. Approximately 1 in 100 BVDU and IFN- α treated HEL cells harboured a virus genome that could be activated in the normal way. Scheck et al. (1986) determined that establishment of latency was not due to intrinsic properties of BVDU because another nucleoside analogue, ACV, also facilitated the establishment of latency after a high multiplicity infection.

It has been shown that during the processes of latency

changes in cellular ultrastructure and viral gene expression occur (Hung et al., 1984). During establishment of latency enveloped HSV-like particles were visible by electron microscopy and polyadenylated (polyA⁺) virus-specific mRNA was readily detectable. However, following inhibitor removal neither viral mRNA or virus morphological structures were apparent. Superinfection of latently infected HEL monolayers with HCMV induced the synthesis of IE gene products and the development of normal nucleocapsids preceded by proliferation of rough endoplasmic reticulum, polysomes and golgi vesicles.

Primary rat and human neurones have also been utilized to establish a high multiplicity latent infection with HSV-1 upon combined inhibitor treatment followed by increased temperature (Wigdahl et al., 1983, 1984b). A repressed HSV infection in HEL cells has also been induced with phosphonoacetic acid, cycloheximide and by use of an UV irradiated virus (Colberg-Poley et al., 1979a; Nishiyama and Rapp, 1981; Shiraki and Rapp, 1986). The synthesis of viral proteins in the HEL latency system in which latency is established in the presence of cycloheximide was apparently confined to the major DNA-binding protein and Vmw110 during the maintenance phase (Shiraki and Rapp, 1989).

Youssoufian et al. (1982) described a persistently infected T-cell line, CEM, in which viral gene expression could be modulated by mitogen treatment. Under normal circumstances the lymphoblastoid cells contained multiple copies of the HSV-1 genome and were productively infected. However, two natural non-productive states occurred in which each cell contained 1-2 copies of the virus genome. Treatment with ~~concanavalin~~ Concanavalin A resulted in a stable latent state that could be reversed by phytohaemagglutinin treatment. During stages of non-production, infectious virus and viral antigens were undetectable.

HSV has been isolated from the ocular tissues of man and experimental animals (Shimeld et al., 1982; Tullo et al., 1985; Cook et al., 1987). Three separate cultures were isolated from rabbit corneas and used to establish an in vitro model for HSV-1 latency (Cook and Brown, 1986, 1987). Rabbit cornea epithelium, keratocyte and endothelium cells

were distinguished on the basis of morphology and indirectly by cell specific markers (Cook and Brown, 1986). All three cell types were capable of supporting a lytic infection, but under non-permissive conditions of temperature elevation, 41.5°C, and infection at very low multiplicity, a latent state could be maintained in these cell cultures (Cook and Brown, 1987). Spontaneous reactivation occurred by downshift to 37°C or was triggered by superinfection of the monolayers with a HSV-1 XbaI mutant (Cook and Brown, 1986, 1987). Treatment of infected cultures with ACV, which eliminates persistent infections, indicated that the host-cell interaction observed appeared to be latency rather than persistence (Cook and Brown, 1987).

Russell and Preston (1986) described an in vitro latency system for HSV-2 based on work originating from Rapp and colleagues. However, this system differed in two important aspects: i) latency was established at low moi in HFL cells at the supraoptimal temperature of 42°C without the use of inhibitors of virus growth, and ii) a stable latent state was maintained at the temperature permissive for virus growth (37°C). Wrzos and Rapp (1987) later adopted a similar approach, but relied upon the use of DNA-negative ts mutants of HSV-1 to establish a latent infection in HEL cells at the NPT (40.5°C) without the use of antiviral agents. Routinely, parental input HSV-2 was reactivated by intertypic superinfection of latently infected HFL cells at the NPT (38.5°C) with ts mutants or with HCMV (Russell and Preston, 1986), notable exceptions to this being adenovirus type 5 (Ad5) and the HSV-1 deletion mutant dl1403 (Russell et al., 1987). This system has been extensively characterized (Russell, 1989) and, importantly, it has been shown that the latent HSV-2 genome is predominately non-linear (Preston and Russell, 1991) which compares with observed phenomena in vivo (Section 1.3.3.2.3.). Thus, despite similarities with the system described by Wigdahl et al. (1981, 1982b) the differences are fundamental because in contrast the latent genome in this system is non-integrated and linear (Wigdahl et al., 1984b; Scheck et al., 1986).

The in vitro latency system described by Russell and Preston (1986) forms the basis of the experimental work presented in this thesis.

1.3.2. Establishment of Latency.

1.3.2.1. Site of HSV Latency.

Although the nature of viruses was not known, a consideration of earlier experimental and clinical findings led Goodpasture (1929) to postulate that latent HSV-1 infections were established specifically within nervous tissues, including the trigeminal ganglia, since these were known to supply sensory enervation to the ophthalmic and orofacial areas in which herpetic lesions were observed to recur.

In subsequent years suggestive evidence supporting this hypothesis was gathered culminating with the direct demonstration of latency in sensory ganglia (Stevens and Cook, 1971). Virus could not be detected in cell free homogenates of nervous tissue but by means of explantation and in vitro cocultivation chronic latent infections were demonstrated in the spinal ganglia of mice (Stevens and Cook, 1971) and the trigeminal ganglia of rabbits (Stevens et al., 1972). Additional supportive evidence was generated by other workers (Knotts et al. ., 1973; Baringer and Swoveland, 1974; Scriba, 1975,1976). Subsequently, Cook and Stevens (1973) demonstrated that following inoculation of virus on the skin, HSV travelled via subserving nerves to the corresponding sensory ganglia. Furthermore, it appeared that latency could be established in any murine sensory nerve ganglia depending on the route of inoculation (Walz et al., 1974) as well as ganglia of the autonomic nervous system (Price et al., 1975a).

By in vitro cultivation of human trigeminal ganglia obtained at post mortem it was possible to recover HSV (Bastian et al., 1972; Plummer, 1973; Baringer and Swoveland, 1973; Rodda et al., 1973). HSV-1 has been isolated from human superior cervical ganglia and vagus ganglia, indicating that, as in experimental mice, virus was

present in the autonomic and somatic ganglia of the natural host (Warren et al., 1978). HSV-2, which infects genital regions, has been shown to reside latently in human sacral ganglia (Baringer, 1974).

HSV has also been detected in the CNS of experimental animals by explantation of brain or spinal cord tissue (Plummer, 1973; Knotts et al., 1973; Cook and Stevens, 1976; Cabrera et al., 1980). Cabrera et al. (1980) showed that 95% of trigeminal ganglia of latently infected mice contained virus that could be recovered by explantation. In comparison, only 5% of brain hemisphere explants were shown to harbour latent virus. However, HSV specific DNA sequences could be detected by liquid hybridization in 30% of the brains of mice that had latent HSV in the trigeminal ganglia implying that explantation provided an underestimate of the amount of latent virus in the CNS, presumably because of differences between the CNS and peripheral nervous system (PNS) and the ability of virus to reactivate in these tissues. Other workers have detected latent HSV DNA sequences in the CNS of mice (Puga et al., 1978; Rock and Fraser, 1983; Stroop et al., 1984) and humans (Sequiera et al., 1979; Fraser et al., 1981).

1.3.2.2. Identification of Latently Infected Cells within the Nervous System.

During an acute HSV infection schwann, satellite and other support cells undergo an abortive infection (Dillard et al., 1972; Schwartz and Elizan, 1973; Cook and Stevens, 1973; Knotts et al., 1974). Limited data suggests that latent virus cannot be recovered from nerve roots, although virus particles pass through them to the ganglia after a peripheral infection, and nerve root tissues are capable of supporting a productive infection in culture (Baringer and Swoveland, 1973; Cook and Stevens, 1973). There is strong evidence, both direct and indirect, to suggest that HSV resides in neurones during latency. A report by Cook et al. (1974) implicating neuronal latency was described in which viral DNA in explanted ganglia was detected by in situ hybridization using a complementary RNA probe. In the same

study, synthesis of viral DNA, antigens and particles in reactivating ganglia occurred in the neurone before detectable spread to surrounding support cells. Similarly, Kennedy et al. (1983) showed that HSV antigen expression was first observed 3 days after dissociation of latently infected dorsal root ganglia in neurones and spread later to fibroblast and schwann cells. Reactivation of a ts mutant stimulated under non-permissive conditions (to prevent the development of secondary foci of infection) by explantation in vitro and neurosurgery in vivo permitted the unambiguous identification of the neuronal body cell as the site for harbouring latent virus (McLennan and Darby, 1980). Recently, viral gene expression in sensory ganglia during the latent phase of infection has been the object of intense study (Section 1.3.5.4). This followed the initial observation by Stroop et al. (1984) in which latency specific mRNA detected by in situ hybridization was shown to be confined to the neuronal nucleus.

1.3.2.3. Extraneural Sites of HSV Latency.

There is a mounting body of evidence indicating that in a variety of experimental systems latent virus may be harboured in cell types other than neurones. In guinea pigs both HSV-1 and HSV-2 have been recovered from the primary sites of infection (Scriba, 1976,1977; Walz et al., 1977; Donnenberg et al., 1980). Scriba (1981) reported that HSV was isolated frequently from inoculated footpad (94%) and vaginal (30%) explanted tissues. However, treatment of explanted peripheral tissues with phosphonacetic acid inhibited the recovery of virus, implying that the phenomena observed by Scriba (1976,1977,1981) were a consequence of a low level productive infection. HSV-1 was recovered from 8% of explants of ear skin taken from latently infected mice in the absence of clinical lesions (Hill et al., 1980). In addition, the prolonged persistence of HSV-1 and HSV-2 was demonstrated in mouse footpads following inoculation at that site (Al-Saadi et al., 1983; Subak-Sharpe et al., 1984; Clements and Subak-Sharpe, 1988; Al-Saadi et al., 1988). ACV chemotherapy provides a means of distinguishing a

persistent HSV infection from a latent infection which is not sensitive to treatment with this drug (Field, et al., 1979; Blyth et al., 1980a; Klein et al., 1981). Extended treatment of mice latently infected with ACV administered in drinking water or combined with intraperitoneal injection did not prevent the recovery of HSV from footpad or ocular tissue following explant and culture (Clements and Subak-Sharpe, 1988; Al-Saadi et al., 1988; Claoué et al., 1990). This suggested that the virus isolated was not derived from a chronic low grade infection but was due to a latent infection. Furthermore, since nucleated neuronal cell bodies are absent from the footpad, then by extrapolation the site of latency must be non-neuronal cells. Latency in extraneural tissues was established independently of sensory ganglia (Scriba, 1981; Al-Saadi et al., 1988; Hill et al., 1983a; Clements and Subak-Sharpe, 1988). Interest has arisen in the type of peripheral cell that could serve as a reservoir of latent virus. Potential candidates in guinea pigs may be vaginal, uterine and cervical epithelial cells, cervical and uterine glandular epithelium and myometrium (Scriba, 1981), although these cell types are probably sites of persistent and not latent infections. Cook and Brown (1987) presented data for the ability of HSV-1 to establish a latent infection in vitro in epithelial, endothelial and keratocytes derived from rabbit cornea. Clements and Jamieson (1989) demonstrated directly by in situ hybridization the cell types in the mouse footpad from which reactivation occurred. HSV specific RNA was detected in the basal and root sheath cells of hair follicles, epithelial cells of sebaceous glands and epidermis cells (Clements and Jamieson, 1989). The relevance of extraneural sites for latency in recurrent human disease is not clear. Cultures of skin explants from sites in humans at which recurrences had been observed did not yield virus (Rustigan et al., 1966). However, recurrent herpetic lesions have been observed in patients with accidentally denervated areas of skin (Hoyt and Billson, 1976). Also, HSV has been isolated from human corneas removed during routine corneal transplantation as therapy for chronic stromal keratitis (Shimeld et al., 1982; Tullo et al., 1985).

1.3.2.4. Axonal Transport.

A number of theories have been considered with regard to the mode by which HSV travels from peripheral sites of inoculation to the sensory ganglia, the CNS and within the CNS (Wildy et al., 1982; Price, 1986). There is overwhelming evidence for transport within axons as the most important route by which virus spreads. Other routes, such as travel by replication in support cells, may have a secondary role. The time interval between initial infection of the mouse footpad and arrival of the virus has been measured (Cook and Stevens, 1973). The translocation rate was estimated to be between 20-24hr, consistent with the rapid movement of macromolecules and organelles by retrograde transport and apparently incompatible with repeated cycles of peripheral replication. Interruption of axonal flow by nerve section or treatment with inhibitors of axonal transport eg. colchicine, blocks viral passage to the ganglion (Wildy, 1967; Kristensson et al., 1971). Virion particles contained within vesicles have been observed in the axoplasm of peripheral nerves by direct electron microscopic examination (Hill et al., 1972; Cook and Stevens, 1973; Baringer and Swoveland, 1974). In cultured rat dorsal root ganglia enveloped virions have been visualized attached to the neurites prior to internalization of nucleocapsids which presumably resulted from viral and cellular membrane fusion (Lycke et al., 1984).

1.3.2.5. Host Factors Involved in Establishment of Latency.

Host responses are important in the pathogenesis of herpetic disease. Some neuronal cells may be non-permissive for HSV replication (Levine et al., 1980). However, if the host is too restrictive the initial acute infection would not occur or recurrence would be aborted. Conversely, if the host fails to restrict viral replication then the period during which virus could spread would be abbreviated. It has been shown that reinoculation of a peripheral site does not permit colonization by a second virus (Klein et al., 1977, 1978; Gerdes and Smith, 1983) which suggested that

local factors produced in response to the initial infection may have prevented a second latent infection. In apparent agreement with these conclusions, analyses of viruses recovered from human ganglia at post mortem with restriction enzymes indicated that a single virus could be recovered from a colonized ganglion (Lonsdale et al., 1979). However, other studies have produced conflicting results (Brown et al., 1979; Lewis et al., 1984; Thomas et al., 1985). Indeed, Meignier et al. (1983) demonstrated that more than one HSV strain could reside in a latent form in the same murine trigeminal ganglion. The course of HSV infections can be modified by host strain, age, dose and route of infection. Increased host resistance favours a latent infection rather than a productive infection (Price et al., 1979). Likewise, the integrity of post ganglionic axons influences the outcome of a herpesvirus infection. Neurectomy performed after the virus arrived at the superior cervical ganglion induced acute phase viral replication (Price et al., 1979). Altered neuronal permissiveness can also be induced in the presence of HSV neutralizing antibody, facilitating the establishment of a persistent HSV infection in rat neurotumour cells in vitro (Doller et al., 1979).

Experimental evidence has suggested that latency may be established very early, within 24hr post infection (Steiner et al., 1990). This may reflect events under natural circumstances and therefore, at the molecular level the host immune system may never play a role in the establishment of latency. However, a body of evidence has accumulated indicating that in animals a range of immune responses are elicited during the primary infection that may restrict the spread of replicating virus. In non-immune mice extensive centrifugal spread of virus back to the eye (the site of inoculation) contributed significantly to the later development of severe ocular damage (Dyson et al., 1987). Tullo et al. (1982) reported that the severity of eye infection correlated with the frequency and site of latency, as the severity of acute infection increased the likelihood of latency in non-ophthalmic branches of the trigeminal ganglia increased. Passive immunization of immunocompetent

mice inoculated at various sites with rabbit hyperimmune serum or human γ -globulin curtailed virus growth and dissemination within the peripheral site of infection and the nervous system and, although the incidence of latent infections was generally reduced, latency was not absolutely excluded (Openshaw et al., 1979; Kino et al., 1982; Shimeld et al., 1990a). In congenitally athymic mice, passive transfer of polyclonal or monoclonal anti-glycoprotein D serum reduced the amount of virus reaching the nervous system but elimination of the virus from the ear pinna of virus required cell mediated immunity (Kapoor et al., 1982). In a study of the effects of prior oral exposure to HSV-1 on the clinical response of animals following subsequent intravaginal inoculation with HSV-2, a reduced percentage of guinea pigs developed genital lesions, although 56% of animals (including some asymptomatic hosts) eventually developed recurrent symptoms (Berkstein et al., 1989). McKendall et al. (1977) suggested that in mice immune to HSV-1 a lower incidence of HSV-2 infection ensued after footpad challenge 3 weeks after the initial infection, in comparison to nonimmune controls. The number of animals harbouring latent virus in uterine tissue was reduced when challenged with the homologous serotype (Walz et al., 1977). Active immunization with live virus prevented CNS fatal involvement although latent superior cervical, lumbrosacral and trigeminal ganglionic infections still developed in the presence of high titres of neutralizing antibody (Price et al., 1975a,b). In summary, it is clearly more difficult to establish a latent infection and such latency is limited when previously infected, passively immunized or vaccinated animals are reinfected.

1.3.3. Maintenance of Latency.

1.3.3.1. Antibody-mediated Immunity and Physiology of the Neurone.

Stevens and Cook (1974) originally proposed that immune mechanisms were important for maintaining HSV latency. Latently infected murine spinal ganglia implanted in

millipore chambers into the peritoneal cavity of a recipient animal, removed four days later and assayed for the presence of viral antigens were more likely to reactivate when implanted into non-immune animals (Stevens and Cook, 1974). Furthermore, the adoptive transfer of anti-herpes immunoglobulin G was as effective as whole rabbit anti-HSV serum in reducing the level of virus (Stevens and Cook, 1974). However, Sekizawa et al. (1980) reported that serum neutralizing antibody against HSV was unnecessary for the maintenance of latent HSV infections. In this report, mice were inoculated with HSV-1 by the lip or corneal route and passively immunized two days later by intraperitoneal injection of rabbit anti-HSV antibodies. Circulating neutralizing antibody declined to undetectable levels two months post infection and 90% of antibody negative mice harboured latent infections in the trigeminal ganglia. In support of the hypothesis that neutralizing antibody is unlikely to effect maintenance, it is known that in humans there is no correlation between observed levels of anti-HSV antibodies and the frequency of recurrent disease (Corey et al., 1980).

Although immune mechanisms could be involved in the control of maintenance, it is likely that the physiology of the neurone is a more important factor. The neurone is a terminally differentiated and highly specialized cell. Many biochemical functions in the neurone are repressed. The permissiveness of neuronal cells to HSV replication is variable and may be influenced by exogenous factors that alter the metabolism of ganglionic cells (Price and Schmitz, 1978). After the neonatal period cell division does not occur in individual neurones, although in cultured neurones in vitro repair synthesis of DNA has been detected (Sanes and Okun, 1972). In latently infected neurones viral DNA is treated like cellular nucleic acids and organized into chromatin (Deshmane and Fraser, 1989) which may facilitate the development of a long term stable relationship between the latent viral genome and the neurone.

1.3.3.2. Organization of the Latent HSV Genome.

1.3.3.2.1. Dynamic versus static latency.

It has been hypothesised that during latency viral DNA could be maintained in cells in a non-replicating form in which virus multiplication is reversibly interrupted (static latency) or in a state that replicates at an undetectable, slow rate that is prevented from inducing a recurrent lesion by components of the host immune system (dynamic latency) (Roizman, 1966). The evidence collected to date does not permit an absolute discrimination between static or dynamic latency.

Baringer and Swoveland (1974) detected by electron microscopic examination of rabbit trigeminal ganglia sections the presence of rare morphologically abnormal neurones that contained viral nucleocapsids. However, this low level of replication may represent spontaneous reactivation of active infection in a minority of latently infected neurones. Using organ culture techniques, Schwartz et al. (1978) reported the isolation of infectious virus from mouse ganglion homogenates.

In contrast, other workers have reported that infectious virus could not be recovered from latently infected ganglia when assayed directly (Stevens and Cook, 1974), and that virus specific antigens or products could not be demonstrated by immunofluorescence or ultrastructural examination of latently infected ganglia (Stevens and Cook, 1971). Furthermore, it is known that DNA-negative ts mutants of HSV can establish latency (Lofgren et al., 1977; McLennan and Darby, 1980; Al-Saadi et al., 1983).

An alternative hypothesis, round-trip latency, suggests that a combination of static and dynamic latency occur. In this case, latency is predominantly static but episodes of reactivation occur that produce virus particles which are transported intra-axonally to the cell surface where following replication the virus infects adjacent nerve termini, thus inducing a latent infection in new neuronal cell bodies (Klein et al., 1976).

Certain lines of evidence suggest that the dynamic and round-trip hypotheses are not operative: i) antiviral therapy using drugs like ACV, which act at the level of viral DNA replication, do not eliminate latent infections arguing against a dynamic maintenance of latent DNA (Field et al., 1979; Blyth et al., 1980b; Field and de Clercq, 1981), ii) in some experimental systems reinfection of ganglia harbouring a latent infection with a second virus is an unusual event (Klein et al., 1977, 1978; Gerdes and Smith, 1983) and iii) in studies on the effect of antiviral treatment of recurrent human herpesvirus lesions, it has been reported that antiviral therapy eradicated ACV-resistant isolates but the variants were not detected in subsequent recurrences implying that the ACV-resistant isolates were unable to re-establish a latent infection (Parris and Harrington, 1982; Ellis et al., 1987) which, together with point ii), suggests that the round-trip mechanism for conservation of the latent genome is not tenable.

1.3.3.2.2. Determination of copy number.

Although it is accepted that the sensory neurone harbours latent HSV (Cook et al., 1974; McLennan and Darby, 1980; Kennedy et al., 1983) an accurate determination of the number of latent genomes per infected neurone has not been made. Quantitative blot hybridization studies have produced several estimates of the number of viral genomes in latently infected cells. Efstathiou et al. (1986) determined that in human trigeminal ganglia 0.01-0.1 HSV copies per cell genome equivalent were present, and 0.78 HSV copies per cell were found in mouse cervical ganglia. Latent HSV DNA has been detected in the CNS of mice, in total brain tissue at between 0.015-0.15 copies (Rock and Fraser, 1983) and at a mean concentration of 0.26 and 0.19 copies in brain stem and spinal cord tissue respectively (Efstathiou et al., 1986). In a tissue culture latency system in which latency was established in the presence of BVDU and IFN- α , latent HSV was retained in HEL cells or rat foetal neurones at 0.25-0.5 or 8-10 copies per haploid cell genome equivalent

respectively (Wigdahl et al., 1984b). Youssoufian et al. (1982) detected 1 copy of the latent HSV genome per cell equivalent in a T-cell line persistently infected HSV. Liquid phase hybridization studies detected 1.2-2.0 and 0.11 HSV copies per cell equivalent in acutely and latently infected murine trigeminal ganglia respectively (Puga et al., 1978).

Infectious centre assays have been used to evaluate the percentage of cells within murine dorsal root ganglia infected with HSV. It was estimated that during the acute phase of infection (6-8 days post inoculation) 1.0% of ganglion cells were infected, after which the percentage declined to approximately 0.1% and remained constant during a period considered to be the latent phase of infection (6 weeks to 15 months post inoculation) (Walz et al., 1976).

Only 5-10% of cells that constitute a ganglion are neurones (Walz et al., 1976). The percentage of neurones that harbour latent infections has been estimated by the reactivation potential of HSV following dissociation of dorsal root ganglia. This method predicts that between 0.2-0.4% of neurones contain reactivatable virus (Walz et al., 1976; Kennedy et al., 1983). The true value is believed to be greater, indeed the percentage of neurones expressing LAT is approximately 3% (Rock et al., 1987a), suggesting that a portion of neurones harbouring latent infections cannot be induced to reactivate following explantation of ganglia, a situation that clearly occurs in the CNS of mice (Cabrera et al., 1980). Moreover, if reactivation is asynchronous, the 0.2-0.4% value may be an underestimate since this pertains to a single time point.

1.3.3.2.3. Molecular biology of the latent HSV genome.

Information on the physical nature of the latent genome is crucial for a complete understanding of the molecular mechanisms involved in the virus-cell interactions that occur during latency. It has been postulated that HSV DNA during latency may be organized in one of a number of states that are illustrated in Figure 1.5. Analysis of the restriction endonuclease cleavage patterns of latent DNA and

Figure 1.5. SCHEMATIC REPRESENTATION OF THE POSSIBLE PHYSICAL ORGANIZATION OF THE LATENT HSV GENOME.

The genome of HSV-1 is a double-stranded DNA molecule that is unit length and linear (Roizman, 1979). The latent HSV-1 (F) genome lacks the terminal restriction fragments BamH1 P and S, and contains a bimolar joint fragment BamH1 PS generated by the fusion of BamH1 P and S. Therefore, the latent HSV genome may be organized into a form that is circular, concatemeric, concatemeric and integrated, or unit length, linear and integrated via regions other than the termini (Rock and Fraser, 1983,1985; Efsthathiou et al. 1986). Current evidence suggests that latent HSV DNA is a circular episome (Mellerick and Fraser, 1987).

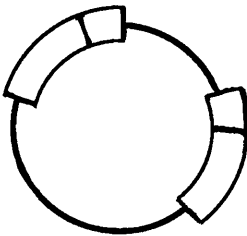
UNIT LENGTH :

1. Linear



ENDLESS :

2. Circular



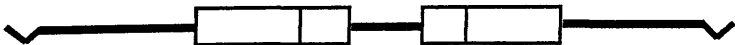
3. Concatemeric



4. Integrated, concatemeric



5. Integrated, linear



comparisons with parental virion DNA has been employed to determine which of the predicted structures reflects that found during latency. DNA molecules that lack terminal restriction fragments have been called "endless" and could be generated from the covalent linking of the termini to form a closed circle or multimer which may or may not integrate into the host genome, or linear and integrated into the cellular genome via regions other than the termini. Alternatively, the latent viral genome could persist in a unit length form similar to that found in virus particles.

Following reverse phase chromatography of digested mouse DNA, Puga et al. (1984) detected terminal fragments of uncharacteristic size implying that either rearrangements of the viral genome had occurred or that viral sequences had integrated randomly into the host genome at a limited number of sites. Alternatively, this result may reflect the purification of cellular sequences that hybridize to homologous regions in the HSV termini (Peden et al., 1982).

In some instances, viral DNA detected in the CNS of normal and neuropathological cadavers was found to contain apparently normal terminal restriction fragments (Fraser et al., 1981). This result resembled that obtained using an in vitro latency system, in which the predominant form of latent DNA isolated from HEL cells or rat foetal neurones retained the terminal fragments suggesting that the latent HSV-1 genome was non-integrated and linear (Wigdahl et al., 1984a).

Rock and Fraser (1983) established a latent infection in mice with HSV-1 by corneal inoculation, and determined that the majority of viral genome was present during latency in the trigeminal ganglia and CNS. However, a fragment composed of the internal repeat sequences did not hybridize to HSV-1 terminal DNA fragments in samples of total mouse brain or pooled trigeminal ganglia, although HSV-1 junction fragments could be detected in these samples. This implied that, unlike previously described data, latent HSV DNA was maintained as an "endless" molecule that was qualitatively different from that found in mature virions. Efsthathiou et al. (1986) confirmed that viral DNA in latently infected mouse tissue was "endless" and extended this analysis to the

examination of unselected human trigeminal ganglia at post mortem. Importantly, latent viral DNA detected in the natural host contained sequences in a similar organization to that found in latently infected mice. Also, EcoRI digestion of latent DNA detected U_L and U_S inversions which provided evidence for the existence of all four isomeric forms of viral DNA (Efsthathiou *et al.*, 1986).

In virion DNA the joint and unique sequences are present in a molar ratio of 1:1. A combination of specific BamHI fragments has been used to compare the relative molarities of joint to unique sequences in DNA extracted from mouse brain tissue latently infected with HSV-1 strain F. The BamHI B fragment hybridizes to two unimolar fragments of the HSV-1 (F) genome, BamHI B and BamHI E, whilst BamHI PS hybridizes to the junction between the unique sequences IR_L/IR_S . Southern hybridization with BamHI B and PS, followed by densitometric analysis, indicated that in latently infected mouse brain tissue the joint region was over represented and present in a molar ratio that approximated to 2:1 relative to BamHI B or E (Rock and Fraser, 1985). Thus, the "endless" DNA noted by Rock and Fraser (1983) seemed to be generated by the joining of termini to form an additional fragment equivalent to the virion joint fragment either by concatemerization or circularization but not by integration via the termini.

Mellerick and Fraser (1987) subjected viral DNA to buoyant density gradient centrifugation which exploits the differences in G+C content of cellular and herpesvirus DNA. Latently infected mouse brain samples contained DNA with distinct viral and cellular peaks enabling the latent HSV DNA to be recovered independently without excessive shearing. A very small proportion of hybridizing DNA could not be separated from the chromosomal peak by repeated centrifugation and probably represented cross hybridization with cellular sequences. Thus, it was proposed that the majority of latent HSV-1 DNA was extrachromosomal and in a circular, compact form that resisted trapping (Mellerick and Fraser, 1987).

The degree of methylation of cytosine bases plays a major role in the control of gene expression (Doerfler *et*

al., 1981). Several drugs are thought to promote hypomethylation of DNA in vitro. Experiments with 5-azacytidine and N,N'-hexamethylene bisacetamide (HMBA) have shown that incorporation of these agents into growth media at explantation increases the rate of recovery and the number of isolates from sensory ganglia of latently infected guinea pigs and mice (Bernstein and Kappes, 1988; Whitby et al., 1987; Stephanopoulos et al., 1988). This type of experiment has suggested a role for methylation in the maintenance of the latent state by down regulation of viral gene expression. Although 5-azacytidine and HMBA prevent methylation of DNA their mode action may be via other mechanisms (Kolalta, 1985; Sheffery et al., 1982; Cohen and Sheffery, 1985; Ramsay et al., 1986). In an in vitro model for latency, a persistently infected human lymphoblastoid cell line was shown by the use of methylation sensitive restriction enzymes to be extensively methylated during non-productive periods (Yousoufian et al., 1982). The examination of the methylation pattern of HSV-1 DNA in latently infected mouse brains revealed that a subset of the potential CG dinucleotides, including those present in the 5' regulatory regions of known HSV genes, were not extensively methylated in vivo (Dressler et al., 1987).

Recently, Deshmane and Fraser (1989) have shown that latent samples contained HSV DNA that was sensitive to digestion with micrococcal nuclease which attacks the linker between nucleosome units generating an oligomeric ladder with a repeat of about 150bp. 80% of the the latent HSV-1 DNA in mouse brain stem tissue was associated with nucleosomes, including the transcriptionally active region of the latent genome, in an arrangement that resembled chromatin (Deshmane and Fraser, 1989). Chromatin formation may be an important factor in the long term stability of the viral genome in the neuronal nucleus during maintenance of the latent state.

1.3.4. Reactivation, Recurrence and Recrudescence of Clinical Disease.

Reactivation of virus in the nervous tissue can arise spontaneously as a consequence of external stimuli. Thereafter, the virus is transported to the periphery where it initiates a lesion in the neurodermatome relating to a particular sensory ganglion (recrudescence) (Wildy et al., 1982). Recrudescence does not automatically follow reactivation. Indeed, HSV-1 has been demonstrated in the skin of mice and guinea pigs without the development of clinical lesions (recurrence) (Scriba, 1977; Hill et al., 1980). Reactivation presumably results in the production of infectious progeny for orthograde export to the periphery. Consequently, it is likely that virus induced and immune-mediated death of the neurone occurs. Each recurrence would be expected to result in neuronal loss. Perpetuation of the number of latently infected cells must rely either on an initial large reservoir of infected cells or a round-trip mechanism (Klein, 1976). Clinical observations in man suggest that denervation and parathesias appear to be insignificant, implying that reactivation must be a limited event in which virus metabolic toxicity is restricted.

1.3.4.1. Recrudescence Disease.

A wide variety of factors are able to induce recurrent herpetic eruptions in man and experimental animals. The most common are exposure to UV light, trauma, fever, hormonal changes, stress and immunosuppression (Wildy et al., 1982; Hill, 1985). Attempts have been made to develop animal models to examine the control of recurrence and recrudescence. One of the most detailed studies has been made by Hill and coworkers using mice (Hill et al., 1975, 1978; Blyth et al., 1976, 1984). The strain of mouse is critical to the success of inducing recurrence or recrudescence and outbred strains have been of most value (Harbour et al., 1981). Recently, Norval et al. (1987) have developed a reproducible murine model for recrudescence disease in which the host was genetically defined at the

major histocompatibility locus, thereby facilitating the study of immunological parameters in recrudescence.

Early experiments demonstrated that UV irradiation of the originally infected ear produced recurrent HSV in the skin of 21% of mice (Blyth et al., 1976). Production of recurrent HSV disease could also be induced by mild trauma in the skin of latently infected mice (Hill et al., 1978; Harbour et al., 1981, 1983). The effects of stripping the skin of the ear of a latently infected mouse include the development of erythema, formation of vesicles and appearance of virus in the skin 2-5 days later at the site of primary inoculation (Hill et al., 1978). Reactivation can be deliberately stimulated by the application of dimethyl sulphoxide (DMSO) at the site of infection (Harbour et al., 1983; Hill et al., 1983a). DMSO caused a low incidence of clinical disease (10%) but virus could be isolated from the skin of 28% of animals, a percentage equivalent to that produced by cellophane stripping of the ear (Harbour et al., 1983). In the same study, DMSO induced the greatest incidence of virus isolated from the ganglia of all the stimuli tested, which implied that DMSO was an efficient ganglion trigger.

The mechanism for reactivation is not clearly understood although various theories have been advanced, namely the ganglion trigger and skin trigger hypotheses. The ganglion trigger theory states that latent viral genomes resident in sensory ganglia are reactivated by a stimulus to the ganglia. The virus proceeds to the epidermal cells via peripheral nerves and induces a skin lesion. However, clinical disease does not develop in every animal in which virus is reactivated and the ganglion trigger theory does not explain how skin stimuli affect ganglia (Blyth and Hill, 1976; Hill et al., 1985). The skin trigger theory postulates that virus is shed frequently from the ganglia to form microfoci of infected epidermal cells which are usually eliminated by host defence mechanisms. These develop into clinically apparent lesions only if the presence of virus coincides with conditions in the skin that favour virus growth (Blyth and Hill, 1976; Hill, 1985). The skin trigger theory is supported by the observation that HSV could be

isolated by organ culture of clinically normal tissue (Scriba, 1977; Hill et al., 1980). Hoyt and Billson (1976) reported that recurrent herpes simplex developed in patients with blow-out fractures that had severed the nerve supply to the area involved, such that lesions must have arisen from virus already present in the skin. Although the results described above are best explained by the skin trigger theory, latency in the cells of the skin cannot be excluded (Section 1.3.2.3.). It is probable that a combination of a ganglion trigger and a skin trigger are required for recurrence of disease.

Prostaglandins are potent mediators of inflammation in the skin and induce reactivation of latent virus when injected subcutaneously onto the site of previous infection (Blyth et al., 1976). It has been suggested that prostaglandins, particularly of the E series, may be candidates for mediating the skin trigger effects (Hill and Blyth, 1976; Blyth et al., 1976).

In experimental animals reactivation can be induced in response to specific stimuli, notably explantation of latently infected ganglia or other nervous tissues (Stevens and Cook, 1971). Homotypic superinfection of explanted human trigeminal ganglia resulted in the recovery of virus identical to the wt virus spontaneously released from other explanted ganglia of the same individual (Brown et al., 1979; Lewis et al., 1984). Furthermore, superinfection of latently infected mice at the original site of inoculation with a heterotypic HSV strain in vivo stimulated the latent endogenous genome to reactivate (Thomas et al., 1985). Experiments with DNA hypomethylating or demethylating agents demonstrated that the recovery of latent HSV-2 and

herpesvirus saimiri (HVS) could be enhanced by the presence of these chemicals in vitro (Whitby et al., 1987; Bernstein and Kappes, 1988; Stephanopoulos et al., 1988; Mossman et al., 1989). Neurectomy has been shown to be a reproducible stimulus for HSV reactivation in humans (Carton and Kilbourne, 1952). Postganglionic nerve section induced reactivation of virus that could be detected by direct homogenization of murine sensory ganglia whereas sham-operated controls failed to yield virus (Walz et al., 1974;

Price and Schmitz, 1978). However, an intact peripheral nerve supply is essential for the development of recurrent herpes simplex lesions in humans and in mice (Carton and Kilbourne, 1952; Hill et al., 1983a).

1.3.4.2. Immune Control of Reactivation and Recurrent Infections.

The incidence of reactivation is increased by immunosuppression. Openshaw et al. (1979) induced reactivation of HSV in a maximum of 70% immunocompetent mice in which cell mediated immunity was suppressed by intraperitoneal administration of cyclophosphamide and/or X-irradiation. Furthermore, reactivated virus was demonstrated in 10-20% of eye homogenates in the presence of high anti-HSV antibody titres, which implied that neutralizing antibody may not prevent reactivation but may restrict the extracellular spread of virus and hence the severity of recurrent disease (Openshaw et al., 1979). Other workers, using corticosteroid treatment and anti-thymocyte serum, did not report an increase in the recurrence rate in the mouse ear model (Blyth et al., 1980a). Shillitoe et al. (1977) reported that cyclical changes in the specific cell mediated immune response to HSV correlated with recurrent herpetic lesions. In patients with recurrent herpes labialis it was observed that circulating lymphocytes that produced macrophage inhibition factor (MIF) were absent or present in reduced amounts and thus insufficient MIF may have predisposed an individual to recurrent disease (Shillitoe et al., 1977). Similar observations have been made in the guinea pig model for recrudescence (Donnenberg et al., 1980). The production of MIF correlated with delayed type hypersensitivity responses in vivo which implied that a defective T-cell function was responsible for reduced immune surveillance (Nash et al., 1981). Alternatively, B suppressor cells which regulate the intensity of delayed type sensitivity reactions to HSV and are present during latency may diminish host immunity (Nash and Gell, 1980).

1.3.5. The Role of Viral Gene Expression During Latency.

The availability of HSV mutants has permitted an analysis of the viral factors involved in latency. The rationale behind this approach is that if the virus fails to initiate a latent infection or cannot be rescued then the mutated gene has an implied role in either the establishment, maintenance or reactivation processes. The HSV genome has a large coding capacity and examination of HSV-1 deletion mutants has shown that many characterized viral genes are not essential for establishment, maintenance or reactivation of latency. These include the IE proteins Vmw12 and Vmw68 (Meignier et al., 1988; Sears et al., 1985b), glycoproteins D and E, and a viral protein kinase (Meignier et al., 1988). Also, the HSV-1 ori_L is not required for latency (Polvino-Bodnar et al., 1987). The analysis of HSV mutants in relation to latency has been augmented by the use of indirect methods aimed at detecting viral gene products during the various stages of latency. Rather than consider these approaches separately the aim of this section is to draw together the available information to answer the following questions: i) does viral replication necessarily precede latency, ii) do incoming components of the virion participate in regulating initiation of latency, and iii) is expression of viral gene products required for initiation of latency or reactivation from the latent state.

The probability of a latent infection occurring increases after symptomatic HSV disease (Harbour et al., 1981). However, full lytic infection in neuronal cells may require a commitment to cell death. Thus, in the nervous system replication of viral DNA could only be tolerated in a limited form. Numerous studies have analyzed the behaviour of HSV-1 and HSV-2 ts mutants in experimental mice which conveniently have a core body temperature (38.5°C) that is non-permissive for virus growth. It has been noted that examples of HSV ts mutants that do not replicate DNA at the NPT still induce a latent infection in mice (Lofgren et al., 1977; McLennan and Darby, 1980; Watson et al., 1980; Al-Saadi et al., 1983). The use of neutralizing anti-HSV antibodies to limit viral replication less clearly defined

the necessity for DNA replication. Intravenous administration of rabbit hyperimmune anti-HSV serum pre or post inoculation did not prevent reactivation of virus in mice (Sekizawa et al., 1980). Independently, Walz et al. (1977) showed that substantial protection developed against a HSV-1 latent vaginouterine infection in passively immunized mice. Systemic or localized treatment of mice with ACV reduced or prevented the establishment of a latent infection in the sensory ganglia (Klein et al., 1979; Field et al., 1979; Field and de Clercq, 1981), although ACV treatment was not effective against a pre-existing latent infection (Field et al., 1979; Blyth et al., 1980b; Field and de Clercq, 1981). Thus, the use of the drug ACV to inhibit viral DNA synthesis suggested that ~~replication at the~~ periphery was a prerequisite for latency. However, ACV does not prevent the establishment of a latent infection in vitro (Wilcox and Johnson, 1988).

The product of the HSV-1 gene product U_L48, Vmw65, is a major structural phosphoprotein located in the tegument (Heine et al., 1974; Marsden et al., 1978). Vmw65 is responsible for the sequence specific transinduction of IE gene expression in combination with other cellular transcription factors (Preston et al., 1988; O'Hare and Goding, 1988). The importance of Vmw65 activity in lytic infections has been demonstrated by the construction and characterization of a HSV-1 mutant in1814 which has a 12bp inframe insertion in Vmw65 such that the polypeptide is unable to form a complex with cellular proteins and therefore IE gene transcription is reduced (Ace et al., 1989). In the mouse eye model for latency, in1814 established a latent infection without detectable replication in the cornea or trigeminal ganglia, which implied that Vmw65, IE gene expression and viral replication were not essential for establishment or reactivation of latency in vivo (Steiner et al., 1990).

To date no HSV gene product has been identified that is required for establishment of latency. Watson et al. (1980) characterized the latency phenotype of a number of HSV-1 ts mutants. Mutant tsI was described as latency-negative by the criteria of $\leq 15\%$ recovery by explantation at 10^5 pfu

infecting dose. However, subsequent studies revealed that this mutant had at least one further ts mutation which affected replication in mouse cells of neuronal origin and consequently affected the rescuer RI-1 (Cook et al., 1986). There is a growing body of evidence to suggest that very little or no viral gene expression is required for establishment of latency (Russell et al., 1987; Steiner et al., 1990; Wilcox et al., 1990, abstracts of the 15th International Herpesvirus Workshop, Washington DC, USA). Furthermore, it has been hypothesised that suppression of IE promoters by a neuronal specific repressor may predispose a virus to latency (Kristie and Roizman, 1988; Kemp et al., 1990).

The role of certain HSV gene products, notably Vmw175, Vmw110, TK and LAT, in the establishment/reactivation of latency have attracted a great deal of individual attention. These are discussed below with respect to their proposed roles in the stages of latency.

1.3.5.1. Vmw175.

On the basis of the latency-negative phenotype of tsK which has a ts mutation in the gene encoding Vmw175, (Watson and Clements, 1978; Preston et al., 1979a,b) it was postulated that at least this IE gene product was essential for initiation or maintenance of the latent state in vivo (Watson et al., 1980). More recently, Leib et al. (1989b) described two non-replicating Vmw175 mutants which were unable to reactivate in a conventional cocultivation assay. In contrast, the mutants tsK and in1411 (which also does not synthesise Vmw175) were not defective for establishment, maintenance or reactivation in a tissue culture latency system (Russell et al., 1987). It is likely that the observed differences in the requirement for Vmw175 between animal and in vitro latency systems is due to complex interactions with the host. For example, replication and survival at the site of inoculation and transport to the ganglia are processes absent in vitro and therefore the requirement for Vmw175 may be obviated. Green et al. (1981) reported the detection of Vmw175, in the absence of late

proteins by indirect immunofluorescence in 95% of rabbits harbouring latent HSV-1. However, it was not clear if the Vmw175 detected was a residual from the initial acute infection or if it was actively synthesised during latent infections of rabbit trigeminal ganglia. It remains possible that the presence of this polypeptide may be a reflection of spontaneous reactivation which is not uncommon in the rabbit model for latency. Indeed, an independent study using a different methodology, the avidin-biotin complex assay, did not detect Vmw175 in the trigeminal ganglia of mice (Pepose *et al.*, 1986).

1.3.5.2. Vmw110.

The deletion mutants dlx0.7, dlx3.1 and dl1403 have been used to examine the importance of the IE polypeptide Vmw110 in latent infections. In the mouse eye model for latency mutants dlx0.7 and dlx3.1 were unable to spontaneously reactivate upon explantation of latently infected trigeminal ganglia. The inability of dlx3.1 to reactivate, but not dlx0.7, was circumvented by inclusion of 200mM DMSO in the explantation growth media or by superinfection of explanted ganglia with a replication defective virus (Leib *et al.*, 1989b). Mutant dl1403 exhibited reduced virulence but retained the ability to establish a latent infection in the sensory ganglia of mice following corneal or footpad inoculation (Clements and Stow, 1989; Leib *et al.*, 1989b). Although Leib *et al.* (1989b) reported that the reactivation profile of dl1403 was unaltered, the rate at which dl1403 reactivated was dependent on the initial dose of virus and host strain (Clements and Stow, 1989). Likewise, Gordon *et al.* (1990b) showed that host species and virus strain differences were important factors that determined whether dlx3.1 could establish a latent infection or spontaneously reactivate *in vivo*. DL1403 established a non-lytic interaction in HFL cells (Russell *et al.*, 1987; Stow and Stow, 1989) but failed to complement and induce reactivation of latent HSV-2 in a tissue culture latency system (Russell *et al.*, 1987). A functional domain of the protein Vmw110 which was crucial

for reactivation of latent HSV-2 in an in vitro latency system has been resolved (Harris et al., 1989). This domain was located within the N-terminal portion of Vmw110 and correlated with a region important for the intrinsic ability of Vmw110 to transactivate gene expression in transient transfection assays (Everett, 1988). In summary, the absence of functional Vmw110 is dispensible for the establishment of a latent infection, neither does the lack of Vmw110 preclude reactivation in vivo. Nevertheless, in vitro latency studies suggest that reactivation in vivo could be triggered by cellular factor(s) that mimic the action of Vmw110. Important in this regard is the finding that a functional Vmw110 was critical for the de novo synthesis of infectious virus particles after transfection of viral DNA (Cai and Schaffer, 1989). In analogy, reactivation from latency is thought to occur in the absence of pre-existing viral proteins and therefore, the data presented by Cai and Schaffer (1989) predicts a role for Vmw110 or a homologous component of the cellular transcription machinery in the process of reactivation.

1.3.5.3. Thymidine Kinase.

HSV-2 RNA mapping to the region encompassing TK has been detected in human tissues (Galloway et al., 1982) and viral specific TK activity has been demonstrated in latently infected animals (Yamamoto et al., 1977). However, these data are not supported by other independent studies in which HSV-1 TK gene expression was undetectable using in situ hybridization or the polymerase chain reaction (PCR) (Stevens et al., 1987; Puga and Notkins, 1987; Deatly et al., 1987; Rock et al., 1987a; Steiner et al., 1988; Lynas et al., 1989a,b). Early studies reported that arabinosyl thymine selected TK⁻ mutants were reduced for neurovirulence and impaired for latency in mice and guinea pigs (Tenser and Dunstan, 1979; Tenser et al., 1979). These findings implied that expression of TK played a major role in the establishment and maintenance of the latent state or in reactivation. A number of defined HSV mutants have been used to determine which of these features of latency is

affected by the absence of TK. The establishment of latency was not compromised by the incorporation of chimeric TK genes into the HSV genome (Sears et al., 1985a). A well characterized TK mutant HSV-1 (F) Δ 305 described by Post et al. (1981) was effective at establishing a latent infection in rabbits but not in a mouse host (Meignier et al., 1988). Recently, two independent reports have described TK⁻ viruses which retained the capacity to establish an efficient latent infection in murine spinal ganglia. However, these mutants were reactivation negative and reduced for growth on primary neuronal cell cultures (Tenser et al., 1989; Leist et al., 1989). The TK⁻ viruses discussed thus far may also have disrupted the promoter or coding sequences of U_L24 which partially overlaps TK. Mutations in U_L24 can reduce the growth of HSV in cultured cells (McGeoch et al., 1988; Jacobson et al., 1989). Efsthathiou et al. (1989) described the use of a defined TK⁻ mutant, lacking 816bp of the TK coding sequences which affect only the TK ORF, in the mouse ear model of latency. This mutant replicated at reduced efficiency in the ears of infected mice and viral specific DNA could not be detected in latently infected mouse neural tissue which is consistent with previous studies using TK⁻ mutants. However, the TK⁻ virus employed by Efsthathiou et al. (1989) was recovered by superinfection of explanted ganglia with wt virus. This is supported by independent evidence in which an ACV resistant HSV-1 mutant, specifically deleted in only the TK gene, established a latent infection as judged by the expression of LAT in the neuronal nuclei (Coen et al., 1989). Moreover, superinfection of the mouse trigeminal ganglia with a replication-incompetent mutant rescued the TK deficient virus (Coen et al., 1989). Therefore, it seems probable that TK is not essential for establishment of a latent infection, rather that the importance of TK expression in HSV latency may be to facilitate the replication of reactivated virus particles in the neurones.

1.3.5.4. Latency Associated Transcripts.

A preliminary attempt to analyse the status of viral gene expression during latency was reliant upon available technology. Puga et al. (1978) examined the effect of large excesses of viral specific RNA, isolated from latently infected mouse trigeminal ganglia, on the reassociation kinetics of an iodinated HSV-1 probe. Using this method, limited transcription from as much as 5% of the viral genome was beyond the levels of detection. As a consequence of insufficient sensitivity Puga et al. (1978) failed to detect virus mRNA, although 0.11 HSV genome equivalents per latently infected ganglion were demonstrable. This suggested that the transcriptional activity of the majority latent genome was severely depressed. In situ hybridization analysis of deparaffinized sections of mouse trigeminal ganglia and brain tissue detected the presence of HSV-1 specific RNA during the latent phase of infection that was limited to the neuronal nuclei (Stroop et al., 1984). Later, in another study using in situ hybridization, Stevens et al. (1987) demonstrated positive signals only when latently infected murine spinal ganglionic sections were hybridized with groups of cloned viral DNA fragments that included the terminal repeats. More refined analysis suggested that the virally encoded mRNA was localized to the region encompassing Vmw110 (Stevens et al., 1987). Furthermore, Northern blot data illustrated the presence of a 2.6kb RNA transcribed from the opposite strand to that encoding Vmw110 (Stevens et al., 1987). Subsequently, other workers reported the detection of a family of transcripts estimated to be present in amounts between 2×10^4 and 2×10^5 molecules per latently infected neurone (Wagner et al., 1988a). These transcripts were termed LAT and have been found in the trigeminal ganglia of mice (Puga and Notkins, 1987; Spivack and Fraser, 1987; Deatly et al., 1987, 1988), rabbits (Rock et al., 1987a; Wagner et al., 1988b; Gordon et al., 1990a) and humans (Croen et al., 1987; Stevens et al., 1988; Krause et al., 1988; Steiner et al., 1988; Wechsler et al., 1988b; Gordon et al., 1990a). A similar pattern of transcription was also noted in the CNS of latently infected

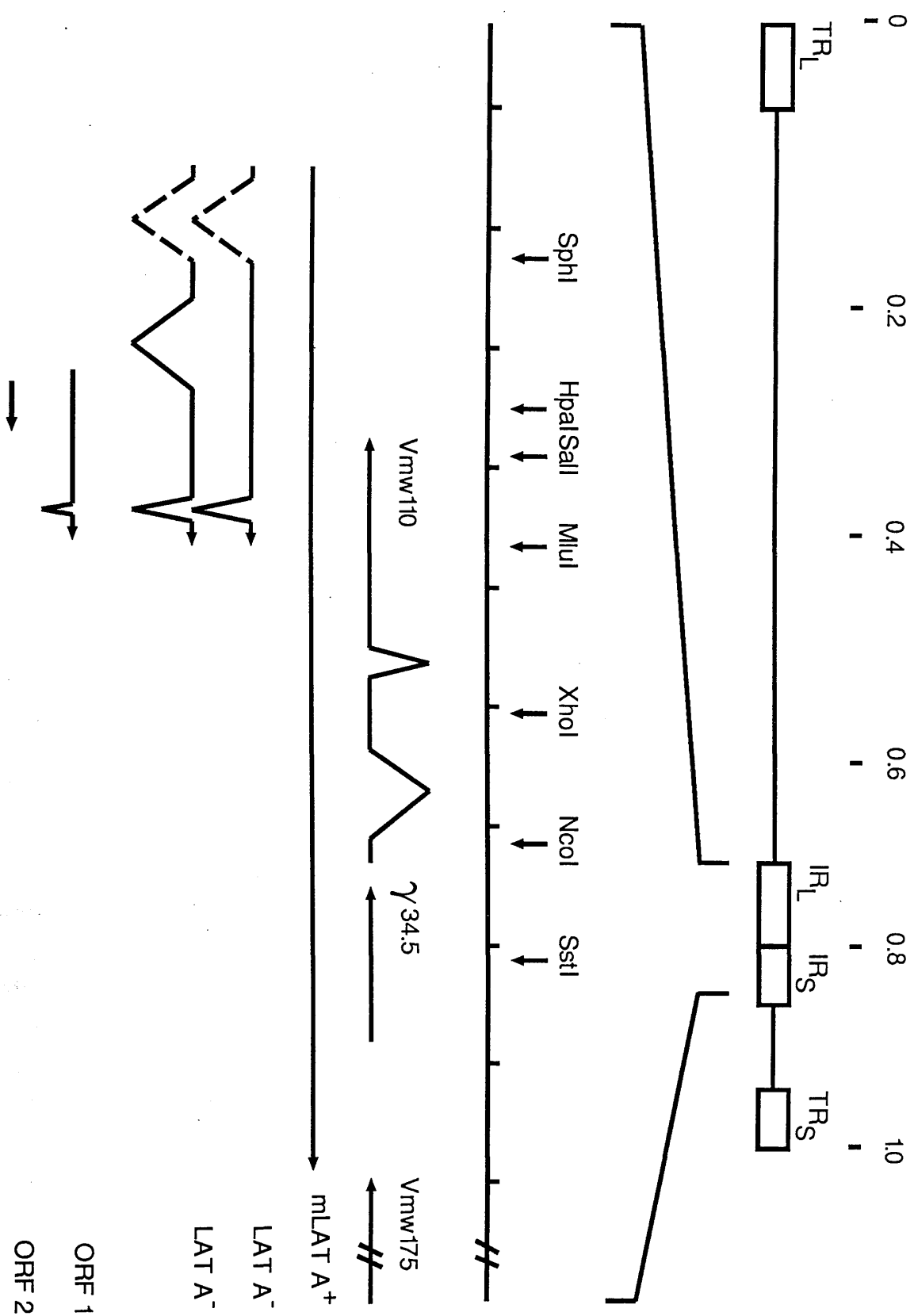
mice (Deatly et al., 1988).

There are at least two LAT species, whose sizes depend on the strain of virus and experimental animal studied. The predominant transcript is approximately 1.8-2.2kb in length and the other less abundant transcript is 1.2-1.5kb in size (Spivack and Fraser, 1987; Rock et al., 1987a; Wagner et al., 1988b; Steiner et al., 1988; Weschler et al., 1988a). During latency LAT has an unexpected subcellular distribution. In latently infected tissue sections a dense in situ hybridization signal was observed overlying the neuronal cell nucleus whereas, in contrast, LAT was detected in both the neuronal cytoplasm and nucleus during a productive infection (Stevens et al., 1987; Rock et al., 1987a; Croen et al., 1987; Krause et al., 1988; Wagner et al., 1988; Steiner et al., 1988; Stevens et al., 1988; Gordon et al., 1990a). Evidence for a polyadenylated LAT species is limited. Puga and Notkins (1987) utilized polyA⁺ RNA isolated from latently infected murine sensory ganglia to direct the synthesis of oligo(dT) primed complementary DNA (cDNA) probes. Furthermore, Spivack and Fraser (1987) reported the isolation of LAT mRNA in an enriched polyA⁺ sample, but transcripts of identical size were also present in a non-polyadenylated (polyA⁻) fraction in amounts comparable to those in a total cell RNA extract. Most LAT in extracts from latently infected mouse or rabbit trigeminal ganglia was recovered in the polyA⁻ fraction upon oligo(dT)-cellulose chromatography and thus appeared not to be extensively polyadenylated (Wagner et al., 1988a,b). It is not clear whether the small amount of polyadenylated LAT detected is a consequence of contamination of polyA⁻ fractions with polyA⁺ RNA or reflects the presence of a minor polyA⁺ species (Figure 1.6.).

Viral RNA expressed during latency in the trigeminal ganglia of mice and rabbits was derived entirely from BamHI B and E HSV-1 (F) fragments (Spivack and Fraser, 1987; Rock et al., 1987a; Figure 1.6.). Analysis of human autopsy material by in situ hybridization confirmed the presence of LAT transcribed from the terminal repeats (Steiner et al., 1988; Stevens et al., 1988; Gordon et al., 1990a). The genomic location of the smaller spliced 1.3-1.5kb message

**Figure 1.6. THE GENOMIC LOCATION OF THE TRANSCRIPTS EXPRESSED
DURING A LATENT INFECTION.**

Illustrated is the physical structure of the HSV-1 genome and beneath a schematic representation of the mRNAs associated with latency in relation to other transcripts arising from the repeat regions. During a productive infection the following transcripts can be detected: IE-1, IE-3, ICP34.5, polyA⁺ mLAT which is up to 8.5kb in length and the approximately 2.0kb larger polyA⁻ LAT. During latency transcription is limited to mLAT, 2.0kb LAT and an additional smaller 1.3-1.5kb LAT. The exact 3' end of LAT is not known. The 3' terminus of mLAT is thought to be 55bp to the left of the polyadenylation signal for IE-3 mRNA. The predicted ORFs encoded by LAT, ORF1 and ORF2, are indicated.



has been shown to be colinear with the 1.8-2.2kb RNA (Wagner et al., 1988b; Spivack and Fraser, 1988a). Wechsler et al. (1988a) proposed the existence of a second splice between nucleotide 1757 to 1847 in rabbits latently infected with HSV-1 McKrae and in human ganglionic sections. Independent examination failed to detect the additional splice 3' of the characterized 730bp intron (Wagner et al., 1988a). Two complementary approaches to the physical mapping of LAT have defined these RNAs in detail (Wagner et al., 1988a; Wechsler et al., 1988a). Wagner and coworkers (1988a) used the mouse footpad model for latency to localise the 5' and 3' limits of the major LAT species of HSV-1 KOS. S1 nuclease and primer extension analysis placed the 5' end of LAT within approximately 500 bases to the left of the KpnI site at 0.783mu and the cap site within the sequence TCCAGGTAGG which is a potential 5' splice signal (Padgett et al., 1986). The 3' end of LAT was mapped to within 310bp to the right of the SalI site at 0.790mu and therefore LAT overlaps the IE-1 transcript by about 1000 nucleotides. The nearest polyadenylation signal for LAT is close to 5.5kb downstream of LAT and just 55bp downstream of the polyadenylation signal for IE-3 (Perry and McGeoch, 1988). LAT RNA in human sensory neurones was indistinguishable from that found in animal model systems (Wechsler et al., 1988b).

Only the larger approximately 2.0kb RNA was present, in reduced amounts, in acutely infected mouse trigeminal ganglia and spinal ganglia (Spivack and Fraser, 1987; Wagner et al., 1988a). Similarly, unspliced LAT has been detected in very low abundance in cultured RS, CV-1 and Vero cells (Stevens et al., 1987; Spivack and Fraser, 1987; Krause et al., 1988). Thus spliced LAT appears to be specific to latently infected neurones.

The suggestion by Spivack and Fraser (1988b) that LAT represented a novel class of HSV-1 genes because LAT was produced in the absence of IE genes (Stevens et al., 1987; Rock et al., 1987a; Spivack and Fraser, 1987) and LAT synthesis was inhibited by cycloheximide and phosphonoacetic acid, remains unconfirmed. To the contrary, in other tissue culture cells the 2.0kb transcript was produced in the presence of a cycloheximide block which implied that LAT

could be expressed like an IE gene in the absence of de novo protein synthesis. (Wagner et al., 1988b).

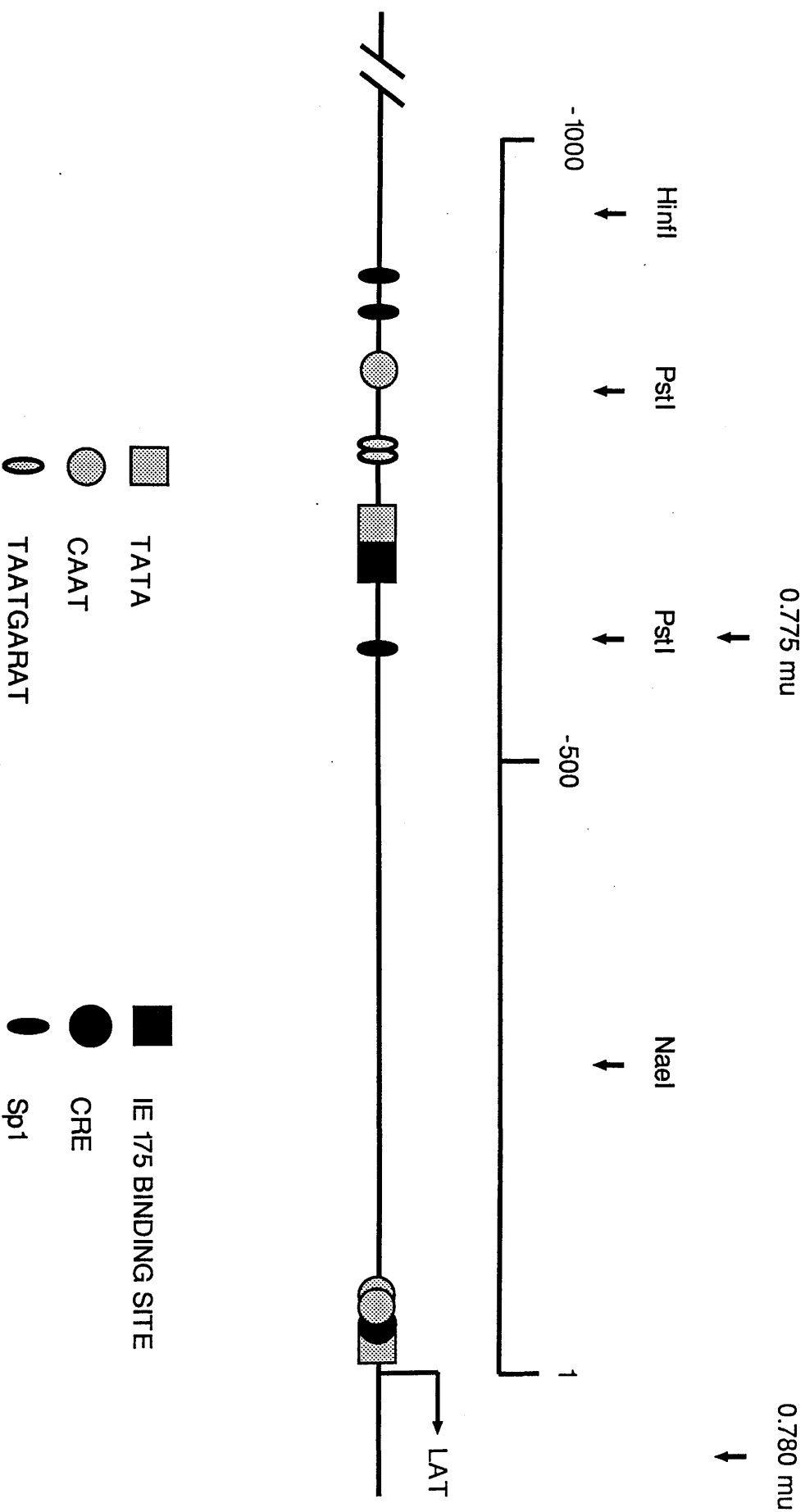
The sequence of the LAT region for three strains of HSV-1 (17 syn+, KOS[M] and F) has been determined (McGeoch et al., 1986; Wagner et al., 1988a; Wechsler et al., 1988a) and on the basis of this two promoter elements and ORFs were suggested (Figure 1.6. and Figure 1.7.). Convention assigned position 1 to the predicted 5' end of LAT which is equivalent to nucleotides 6897 and 11947 in BamHI E and B respectively (by the numbering system of McGeoch et al., 1988).

Examination of the sequences 5' of the proposed LAT cap site indicated the presence of a RNA polymerase II promoter which had weak homologies for a TATA box and two CAAT boxes located at positions -25, -51 and -56 respectively (Wagner et al., 1988a). However, Wechsler et al. (1988a,b) noted a putative promoter present further upstream which was comprised of a potential TATA box, CAAT box, two poor TAATGARAT motifs and three GC rich tracts or SpI binding sites. The second candidate eukaryotic promoter was atypical in that it was located about 700bp upstream of the 5' end of LAT. Transfection assays demonstrated that DNA fragments containing the second promoter element (not the minimal promoter immediately 5' to the nominal start of LAT) were most efficient at directing the transcription of a heterologous gene (Zwaagstra et al., 1989; Batchelor and O'Hare, 1990). Dobson et al. (1989) constructed a recombinant virus that had the β -globin gene under the control of the putative LAT promoter and which produced a polyadenylated, cytoplasmic β -globin mRNA in latently infected murine spinal ganglia. Also, deletion of a 200bp PstI fragment, which contains the predicted TATA box homology and 90bp 3' to the expected cap site, obviated expression of LAT during the latent phase of infection (Dobson et al., 1989).

In summary, these results provided evidence that transcription during latency was regulated by a promoter located about 700bp upstream of the proposed 5' end of LAT. Therefore, production of LAT is either under unique transcriptional control or the sequences between the

Figure 1.7. DNA SEQUENCE ELEMENTS UPSTREAM OF LAT.

Sequence analysis indicated the presence of an RNA polymerase II promoter element 5' of the LAT cap site which has weak homologies for a TATA box and 2 CAAT boxes (Wagner et al., 1988a). However, the functional LAT promoter is located, uniquely about 700bp upstream of the 5' end of LAT and consists of TATA and CAAT box homologies; TAATGARAT and Sp1 motifs which seem to be unimportant in vitro; a Vmw175 binding site and a CRE which regulate LAT constructs in tissue culture; neuronal specific sequences located between nucleotides -797 and -1267 relative to the LAT start site (Zwaagstra et al., 1989,1990; Batchelor and O'Hare, 1990; Leib et al., 1990)



promoter elements and the cap site are an unstable, undetected intron.

The functional dissection of the putative LAT promoter by transient transfection experiments suggested that LAT was controlled by a relatively strong orientation dependent promoter and that deletion of the SpI sites or CAAT box did not significantly reduce CAT gene activity. Also, the LAT promoter was not stimulated by Vmw65, which suggested that the TAATGARAT motifs were not functional (Zwaagstra et al., 1989; Batchelor and O'Hare, 1990). Batchelor and O'Hare (1990) demonstrated that the LAT promoter was negatively regulated by the protein encoded by IE-3. Deletion of a 55bp region that removed a potential binding site for Vmw175 eliminated the activity of the LAT promoter in CAT assays in HeLa cells. In gel retardation assays Vmw175 binds to the LAT promoter region containing the consensus sequence ATCGTCNNNNGCGRC. However, this does not correlate with independent observations that Vmw175 neither stimulated nor repressed expression from the LAT promoter, although it remains possible that the constructs used in this study disrupted the Vmw175 binding site (Zwaagstra et al., 1989).

The LAT promoter is selectively active in cells of neuronal origin due to a cis-acting signal located between nucleotides -797 to -1267 (Batchelor and O'Hare, 1990; Zwaagstra et al., 1990). A cellular factor found in mouse neuronal and non-neuronal nuclei has a specific binding affinity for this cis-domain (Zwaagstra et al., 1990, abstracts of the 15th International Herpesvirus Workshop, Washington DC, USA). A single element essential for expression of eukaryotic genes in neuronal tissues has not been demonstrated (Bray et al., 1988; Johnson et al., 1989; Danciger et al., 1989).

In analogy with HSV, the genomes of two other α -herpesviruses, bovine herpes virus type 1 (BHV-1) and pseudorabies virus (PRV), are transcriptionally active during latency (Rock et al., 1986; Rock et al., 1988). It is interesting to note that BHV-1 LAT maps to a 1.9kb HindIII D region which encodes at least one IE gene and that LAT expression is controlled by an orientation dependent promoter active in primary rabbit sensory neurones (Rock et

al., 1987b; Jones et al., 1990).

Recently it was demonstrated that the LAT promoter was differentially sensitive to extracellular signals such that altered regulation of LAT transcription may have an important role in the reactivation of latent virus. A 7bp consensus cAMP response element (CRE), TGCGTCA, located at position -38 in the LAT region appeared to be functionally significant (Leib et al., 1991). Chemicals that raise cAMP levels positively stimulated the LAT promoter in transfected rat pheochromocytoma cells, which contain a factor that binds specifically to the CRE, and induced the reactivation in vitro of viruses possessing intact LAT but not a LAT-negative (LAT⁻) mutant (Leib et al., 1991). Reactivation of latent HSV-1 in rat sensory neurones in vitro was inducible by stimulation of cAMP dependent pathways with a variety of pharmacological agents (Smith et al., 1990, abstracts of the 15th International Herpesvirus Workshop, Washington DC, USA). The BHV-1 LAT promoter was negatively effected by the synthetic glucocorticoid dexamethasone (Jones et al., 1990). Corticosteroid treatment induced reactivation of BHV-1 in the natural host and in an experimentally infected animal model (Sheffy and Davies, 1972; Rock and Reed, 1982).

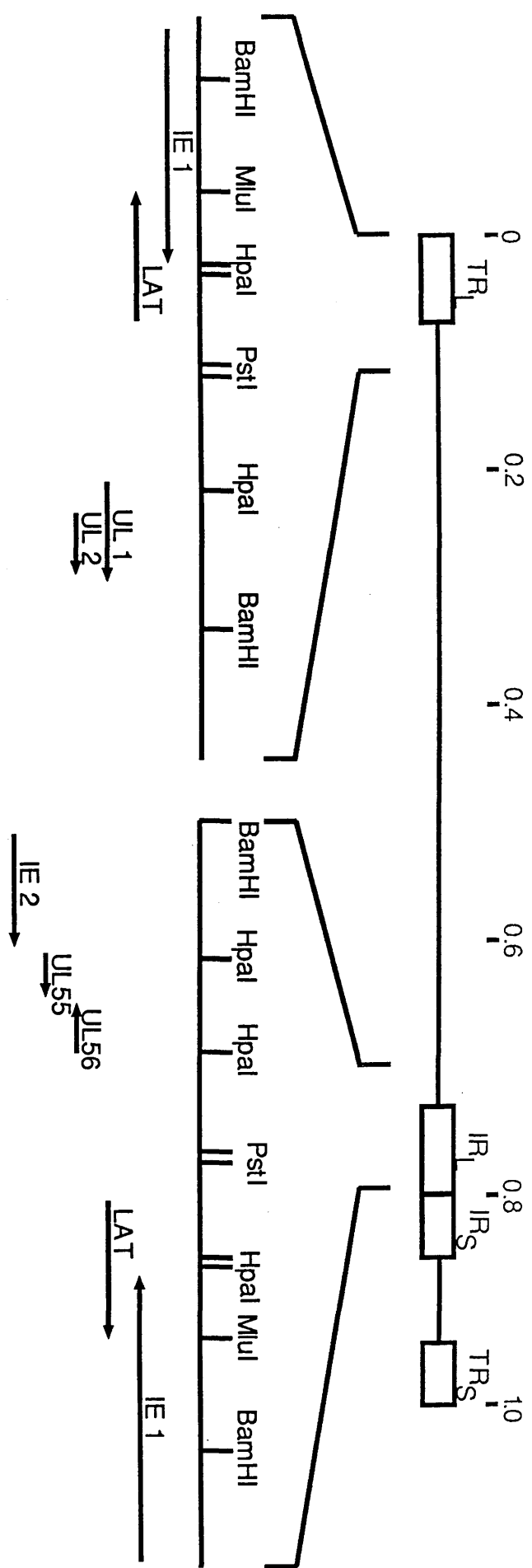
Two significant ORFs were predicted by computer analysis of the LAT genomic sequence (Wechsler et al., 1989). ORF1 and ORF2 have a theoretical coding capacity of 36kd and 12kd respectively. ORF2 is a single exon within ORF1 but in a different reading frame. Antibodies raised against synthetic peptides from these ORFs were unable to detect a LAT protein (Wagner et al., 1988a; Wechsler et al., 1989) which suggested that LAT was not protein coding. Furthermore, direct sequence analysis of a PCR product produced by the amplification of LAT sequences from total cell RNA extracted from murine trigeminal and cervical ganglia latently infected with HSV-1 strain SC16 was found to contain several base changes and deletions with respect to the published HSV-1 strain KOS(M) and 17 sequences (Lynas et al., 1989b). These mutations or deletions in SC16 introduced termination signals within the putative ORF2, suggesting that LAT does not function through translation to a protein. Comparison of HSV-1 and HSV-2 LAT specific

sequences revealed insignificant conservation and atypical codon usage by triplet periodicity tests (McGeoch et al., 1990, abstracts of the 15th International Herpesvirus Workshop, Washington DC, USA). Additional evidence that the LAT regions of α -herpesviruses are widely divergent comes from the fact that the region of the BHV-1 genome that is transcriptionally active during latency and HSV-1 LAT regions do not cross hybridize (Rock et al., 1987b). Moreover, it is also known that LAT accumulates in the nucleus and is not extensively polyadenylated. The mapped 5' terminus of LAT is at a splice donor signal and a splice acceptor consensus lies 1.95kb downstream. Thus, LAT has the properties of an intron excised from a much larger transcript. Indeed, recent evidence has demonstrated that the LAT gene was processed as an intron. The potential LAT intron was cloned into the β -galactosidase gene and Northern blot analysis of transcripts produced in transfected cells detected a correctly sized 3.5kb β -galactosidase and 2.0kb LAT RNA, indicating that LAT was spliced from the primary transcript generating an intact β -galactosidase transcript (Farrell et al., 1991). This result combined with PCR analysis of the LAT species termini, provide evidence that the consensus splice sites are functional in transfection assays. Furthermore, the LAT intron reduced Vmw110 transactivation of the TK promoter by 50-80% in co-transfection assays utilizing a vector containing the LAT region DNA fragment, including the splice signals, cloned into the β -galactosidase gene (Farrell et al., 1991). The importance of LAT inhibition of Vmw110 activity during latency remains to be elucidated, but it has been speculated that nuclear accumulation of the stable LAT intron may act as an antisense mRNA which interferes with Vmw110 function thereby suppressing full viral gene expression during reactivation events.

HSV mutants that abrogate LAT expression have been used to examine the biological relevance of LAT during the establishment, maintenance and reactivation processes of latency. The extents of these mutations are summarised in Figure 1.8. To date all LAT mutants studied retained the ability to establish and maintain a latent infection in the

Figure 1.8. LOCATION OF THE MUTANT VIRUSES USED IN STUDIES OF LAT FUNCTION.

The HSV-1 prototype genome arrangement is shown with BamH1 B and E enlarged. The position and direction of synthesis of the IE-1, LAT, U_L1, U_L2, IE-2, U_L55, and U_L56 mRNA are illustrated. Beneath are the extent of the deletions of the five mutant viruses described in the text. X10-13 is a HSV-1 x HSV-2 recombinant predominately HSV-1 except between the region 0.396-0.404mu which was derived from HSV-2, and spontaneously deleted between 0.019-0.076mu and 0.743-0.808mu (Javier et al., 1988). 1704 is a HSV-1 variant which is deleted in TR_L/U_L and IR_L/U_L (MacLean and Brown, 1987). The exact end points of the deletions are unknown but the deletions are about 1.2kb and 3.8kb in TR_L/U_L and IR_L/U_L respectively. Mutant dlLAT1.8 has a 1.8kb deletion in the LAT sequences between a PstI and HpaI site which removes the putative LAT promoter and 1015bp of the sequences specifying LAT (Leib et al. 1989a). 8117 is a mutant virus deleted for 950bp in TR_L and IR_L between 0.042-0.048mu and 0.778-0.784mu (Izumi et al., 1989). The HpaI P fragment of HSV-1 (F) is not present in the genome of strain HFEM indicating a deletion that spans the co-ordinates 0.762-0.790mu (Rosen and Darai, 1985). TB1 is derived from HFEM and contains an additional 440bp segment of bacteriophage lambda DNA in place of a 168bp deletion in the transcribed portion of LAT (Block et al., 1990).



X10-13		HSV-1xHSV-2
1704		17 ⁺
dLLAT1.8		KOS
8177		KOS
TB1		HFEM

absence of detectable LAT gene expression (Javier et al., 1988; Leib et al., 1989a; Sedarati et al., 1989; Steiner et al., 1989). Defined LAT⁻ deletion mutants (dlLAT1.8 and 8117) and a LAT⁻ mutant (1704) reactivated with reduced efficiency upon explantation and cocultivation of latently infected ganglia, although similar amounts of DNA reached the ganglia compared to wt or rescued viruses (Leib et al., 1989a; Sedarati et al., 1989; Steiner et al., 1989; Mitchell et al., 1990b). Mutant TB1 is an engineered virus that does not produce full length LAT but reactivated with normal kinetics and therefore defined a 168bp region not involved in the slow reactivation phenotype (Block et al., 1990). The reactivation profile of the HSV-1xHSV-2 recombinant X10-13 has been examined in the rabbit eye model in which reactivation occurs spontaneously or is inducible by iontophoresis of epinephrine (Hill et al., 1990). In accord with results described above, expression of LAT had little effect on the frequency of spontaneous or in vitro recovery of X10-13 but the percentage of rabbits induced to shed virus in vivo that reactivated X10-13 was reduced and statistically different from those rabbits infected with a LAT-positive (LAT⁺) rescued derivative of X10-13 (Hill et al., 1990).

A minor species of LAT (mLAT) present in 0.3% of neurones was detected by in situ hybridization of rabbit, mice and human ganglionic tissues using the HSV-1 BamHI SP fragment (Spivack and Fraser, 1987; Rock et al., 1987a; Deatly et al., 1988; Krause et al., 1988; Steiner et al., 1988). Mitchell and coworkers (1990a) analyzed the extent of transcription of mLAT in latently infected mice by probing ganglionic sections with subclones of BamHI B and SP. At most 8.5kb of DNA was transcribed rightwards through LAT, IE-1, γ 34.5 and terminated at a polyadenylation signal close to the 3' end of IE-3 sequences in IRs (Dobson et al., 1989; Mitchell et al., 1990a). An 8.5kb transcript was detected by Northern blot hybridization of a polyA⁺ extract from productively infected cells (Dobson et al., 1989). Recently, a transcript estimated to be between 8 and 9kb in size was detected in the trigeminal ganglia of latently infected rabbits (Zwaagstra et al., 1990). Failure to

produce mLAT did not preclude establishment or maintenance of a latent infection (Mitchell et al., 1990b).

The available data suggest that the HSV LAT region may have a dual function. Firstly, the LAT intron may act as a biologically stable antisense inhibitor of Vmw110 mRNA synthesis, or it may sequester Vmw110 transcripts in the neuronal cell nucleus rendering them inaccessible for translation, or LAT may act on Vmw110 itself preventing activation of HSV gene expression. Secondly, the finding that LAT⁺ viruses generally have a selective reactivation advantage is most easily understood if a portion of the polyadenylated mLAT that extends beyond the LAT intron encoded a polypeptide. There is minimal evidence to suggest that the LAT region produces a functional mRNA encoding a protein. However, preliminary immunocytochemistry using rabbit antiserum raised against a LAT fusion protein points to the existence of a LAA in vitro (Doerig et al., 1990, abstracts of the 15th International Herpesvirus Workshop, Washington DC, USA). External stimuli that increase LAT promoter activity may provide the link between a LAA and reactivation.

2. MATERIALS.

2.1. Virus Stocks.

HSV-2 wt strain HG52, HSV-1 wt strain 17 and HSV-1 mutant tsK (Marsden et al., 1976) were obtained from stocks held in the Institute of Virology and maintained by M. Murphy. Dr. C.M. Preston provided UV tsK, irradiated to reduce the titre at 31°C by a factor of 5×10^5 and obviate gene expression (Notarianni and Preston, 1982). Mutant tsK plaqued with syncytial morphology. HSV-1 deletion mutants dl1403 (Stow and Stow 1986), FXE and D14 (Everett, 1989) were provided by E.C. Stow and Dr. R. Everett. The insertion mutant in1814 (Ace et al., 1989) was provided by Dr. C.M. Preston.

The adenoviruses Ad5 and the recombinants AdMLP-110 and Ad110-110 (Zhu et al., 1988) were supplied by Dr. R. Hay and Professor S. Silverstein respectively. The Ad5 deletion mutant dlE1,3 was a gift of Dr. F. Graham.

HCMV strain AD169 was provided by Dr. J. Russell.

2.2. Tissue Culture Cells.

BHK-21 clone 13 cells (Macpherson and Stoker 1962) were used for the growth and titration of HSV. HFL monolayers were used to establish a latent infection in vitro as described by Russell and Preston (1986). HFL cells were originally provided by Dr. B. Carritt, although latterly Flow 2002 cells provided by Flow Laboratories were used. The 293 cell line established by Graham et al. (1977), was used to propagate and titrate adenovirus stocks.

2.3. Tissue Culture Media.

All cell types were propagated in Glasgow Modified Eagles Medium (Busby et al., 1964) supplemented with 0.25% sodium bicarbonate, 4mM L-Glutamine, 100 units/ml penicillin, 100µg/ml streptomycin and modified as indicated below:

ETC10	10% new born calf serum (Gibco), 10% tryptose phosphate broth.
EF10 & EF5	10% or 5% foetal calf serum (Gibco), 1% non-essential amino acids (Gibco).
EHu5 & EHu2	5% or 2% human serum (Flow Laboratories).

For titrations of adenovirus stocks, monolayers were overlaid with Eagles agar overlay medium, without phenol red, containing 0.65% Noble agar, 0.02% calf serum and 12.5 μ M MgCl₂. Other media routinely used are as follows:

Phosphate buffered saline (PBS) A	170mM NaCl, 3.4mM KCl, 1mM Na ₂ HPO ₄ , 2mM KH ₂ PO ₄ and buffered to pH7.2.
PBSB	6.8mM CaCl ₂ .
PBSC	4.9mM MgCl ₂ .
PBS	8 parts PBSA : 1 part PBSB : 1 part PBSC
Tris-buffered saline	140mM NaCl, 10mM KCl, 10mM Tris-HCl, 1mM MgCl ₂ , pH7.0.
Tris-saline	140mM NaCl, 30mM KCl, 280mM Na ₂ HPO ₄ , 1 mg/ml glucose, 25mM Tris-HCl (pH7.4), 0.001% (w/v) phenol red, 100 units/ml penicillin, 100 μ g/ml streptomycin.
Trypsin	0.25% (w/v) trypsin (Difco) dissolved in Tris-saline.
Versene	0.6M EDTA in PBSA containing 0.0015% (w/v) phenol red.

2.4. Host Bacteria for Plasmid Propagation.

E. coli strain K12 DH-1 (rec A1, nal A, r R⁻, m R⁻, endo I⁻, B⁻, rel A1) was used throughout for plasmid amplification (Hanahan, 1983).

2.5. Bacterial Growth Media.

Bacteria were routinely grown in suspension in L-Broth [1% (w/v) NaCl, 1% (w/v) Bactopeptone, 0.5% (w/v) yeast extract] or on L-Broth agar plates [L-Broth plus 1.5% (w/v) agar], supplemented with 50µg/ml ampicillin as required.

2.6. Plasmids.

Genomic libraries of cloned HSV DNA fragments were established in the Institute of Virology and clones were grown from seed stocks supplied by Dr. C.M. Preston.

2.7. Enzymes.

Restriction endonucleases were obtained from Bethesda Research Ltd. and Boehringer Mannheim. Bethesda Research Ltd. also supplied the enzymes DNA polymerase I and Moloney Murine Leukaemia Virus (MMLV) RNase H⁻ reverse transcriptase. Lysozyme, proteinase K, bovine pancreas RNase A and DNase I were from Sigma Chemical Company Ltd. Boehringer Mannheim provided DNA polymerase (large fragment) and T₄ polynucleotide kinase. Perkin-Elmer Cetus supplied recombinant Thermus aquaticus (Taq) DNA polymerase. RNase-free DNase came from the Promega Corporation.

2.8. Reagents.

Chemicals used were of the highest purity available and obtained from the following suppliers listed below:

BDH Chemicals, Beecham Research Laboratories, Bethesda Research Laboratories, Bio-Rad Laboratories, Fluka, Kochlight Ltd, May and Baker, Melford Laboratories, Merke

Sharp and Dohme International, Pharmacia Chemicals, and Sigma Chemical Company Ltd.

2.9. Radiochemicals.

Amersham International Plc supplied all the radiochemicals used throughout. Specific activities were 40-60 Ci/mmol for [³H]-thymidine, >800 Ci/mmol for [³⁵S]-methionine, 3000 Ci/mmol for α-[³²P]-dNTPs, and >5000 Ci/mmol for γ-[³²P]-dATP.

2.10. Oligonucleotides.

Oligonucleotides were synthesised using a Biosearch 8600 DNA Synthesiser by Dr. J. McLauchlan. Sequences of the oligonucleotides employed are listed below :

LAT 1	5'- GCGCTCGCGGAAACTTAACACCACC -3'
LAT 2	5'- GGGCAGGCTCTGGTGTTAACCACAG -3'
TK 1	5'- CATAACGACGATCTGCGACC -3'
TK 2	5'- CGTCATAGCGCGGGTTCCTT -3'

2.11. Commonly Used Buffers.

TE	10mM Tris-HCl, 1mM EDTA (pH7.5).
TEN	5mM Tris-HCl, 150mM NaCl, 5mM EDTA (pH7.5).
TNE	20mM Tris-HCl, 100mM NaCl, 1mM EDTA (pH7.5).

2.12. Electrophoresis Buffers.

10 x E buffer	36mM Tris, 30mM NaH ₂ PO ₄ , 1mM EDTA (pH7.8).
10 x electro-elution buffer (EEB)	5mM sodium acetate, 1mM EDTA, 40mM Tris-HCl (pH7.8).

10 x TBE buffer	90mM Tris, 1mM EDTA, 90mM boric acid (pH8.3).
Gel-elution buffer (GEB)	250mM ammonium acetate, 20mM Tris-HCl (pH7.4), 2.5mM EDTA.
5 x dye-ficoll loading buffer	10% (w/v) ficoll, 0.1M EDTA, 5 x E buffer, 0.1% (w/v) bromophenol blue.
5 x TBE loading buffer	50% (v/v) 1 x TBE, 1% (w/v) SDS, 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue.

2.13. Lysis Buffers.

Cell lysis buffer	20mM Tris-HCl, 0.5% SDS, 2.5mM EDTA (pH7.5), 0.25mg/ml proteinase K.
Guanidinium thiocynate (GSCN) buffer	4M GSCN, 50mM Tris-HCl (pH7.5), 0.5% (w/v) N-lauryl sarcosine, 2mM EDTA (pH7.5), 100mM β -mercaptoethanol, 0.33% (v/v) antifoam A.
TK lysis buffer	20mM Tris-HCl (pH7.5), 2mM $MgCl_2$, 10mM NaCl, 0.5% (v/v) Nonidet P40.

2.14. PCR Buffers.

DNase buffer	40mM Tris-HCl (pH7.9), 10mM NaCl, 6mM $MgCl_2$.
10 x PCR buffer	100mM Tris-HCl (pH8.2), 0.5M KCl, 15mM $MgCl_2$.
5 x reaction buffer	250mM Tris-HCl (pH8.3), 375mM KCl, 15mM $MgCl_2$.

2.15. Polypeptide Electrophoresis Buffers.

3 x boiling mix	50mM Tris-HCl (pH6.7), 0.4% SDS, 30% (v/v) glycerol, 5% β -mercaptoethanol, 0.1% (w/v) bromophenol blue.
Gel buffer	1.5M Tris-HCl (pH8.9), 0.4% SDS.
Stacking gel buffer (SGB)	0.5M Tris-HCl (pH6.7), 0.4% SDS.
Tank buffer	52mM Tris, 53mM glycine, 0.1% SDS.

2.16. Southern Transfer and Hybridization Solutions.

50 x Denhardts solution	1% (w/v) ficoll, 1% (w/v) bovine serum albumin (BSA) fraction V, 1% (w/v) polyvinylpyrrolidone.
20 x SSC	3M NaCl, 0.3M sodium citrate.
Transfer solution	0.4M NaOH, 0.6M NaCl.
Neutralizing solution	0.5M Tris-HCl (pH7.0), 1M NaCl.
Prehybridization mix	6 x SSC, 5 x Denhardts, 0.1% SDS, 18 μ g/ml, denatured and sonicated calf thymus DNA.
Hybridization mix	20mM Tris-HCl (pH7.5), 7xSSC, 10 x Denhardts, 1mM EDTA, 0.6% SDS, 60 μ g/ml denatured and sonicated calf thymus DNA.

2.17. Radiolabelling Solutions.

Kinase buffer	70mM Tris-HCl (pH7.6), 10mM MgCl ₂ , 5mM DTT.
10 x Nick	0.5M Tris-HCl (pH7.5), 100mM MgCl ₂ , 1mM

translation DTT, 500 μ g/ml BSA.
buffer

Oligo reaction 250mM Tris-HCl and 25mM MgCl₂ buffered to
mix pH8.0, 50mM β -mercaptoethanol, 1M Hepes
 (pH6.6), 30 OD units/ml pd(N₆) in TE.

2.18. Other Solutions

Destain 5% methanol, 3.5% glacial acetic acid.

Fix 1% glacial acetic acid, 9% methanol.

Giemsa 1.5% (v/v) suspension of giemsa stain in
 glycerol heated to 56°C for 2hr and
 diluted with an equal volume of methanol.

3. METHODS.

3.1. Cell Culture.

HFL cells were grown in EF10 in 175cm² plastic culture flasks from a seed stock of approximately 1.5×10^7 cells at 37°C in an atmosphere of 5% CO₂ and 95% air. HFL monolayers were passaged every 3-4 days and harvested by addition of 10ml 1:1 mix of versene:trypsin followed by 10ml of fresh EF10. A homogeneous cell suspension was used to seed subsequent monolayers.

A confluent monolayer of HFL cells in a 35mm or 50mm diameter plastic petri dish yielded approximately 10^6 and 3×10^6 cells respectively.

293 cells were handled in the same manner except that 10ml of versene only was used to detach the cells from plastic surfaces.

BHK cells were grown in ETC10 from a seed stock of about 10^7 cells in an 850cm² plastic roller bottle at 37°C in an atmosphere of 5% CO₂ and 95% air. Cultures were passaged every 2 days as described for HFL cells.

A confluent monolayer of BHK cells in a 35mm diameter dish contained on average 10^6 cells.

Note: HFL, BHK and 293 cells were split 1:4, 1:10 and 1:4 respectively

3.2. Virus Stocks.

3.2.1. Herpes Simplex Virus.

3.2.1.1. Preparation of a Stock.

Confluent BHK monolayers in 850cm² plastic roller bottles were infected with virus at 0.01 pfu/cell in 100ml ETC10. Cells were incubated at 37°C for 3-4 days when extensive cpe was apparent. The bottles were shaken to disperse cells into 20ml of medium and centrifuged at 2,000 revolutions per minute (rpm) for 2hr at 4°C in a Sorvall GSA rotor. This procedure separated the virus preparation into cell released virus (supernatant) and a pellet of virus associated with cells. The pellet was sonicated in 10ml ETC10 and recentrifuged at 2,000 rpm for 15min in a Beckman

GPR centrifuge to remove cell debris. The cell released virus in the supernatant was centrifuged at 12,000 rpm in a Sorvall GSA rotor for 2hr at 4°C and the pellet resuspended in ETC10.

All viral stocks were sterility tested on blood agar plates and stored at -70°C in small aliquots.

3.2.1.2. Titration of a HSV Stock.

HSV stocks of virus were titrated on 35mm petri dishes of confluent BHK monolayers. Serial 10-fold dilutions were made in ETC10 and 200µl of each dilution was used to infect a monolayer (usually in duplicate) from which the medium had been aspirated. Following adsorption at 37°C for 1hr, cells were overlaid with EHu2 or EHu5 and incubated at the temperature permissive for virus replication for up to 3 days. The medium was removed and cells stained with giemsa at room temperature for 30min. Plaques were counted on rinsed plates under a dissecting microscope and the number of pfu/ml determined.

3.2.2. Adenovirus.

3.2.2.1. Preparation of a Virus Stock.

Monolayers of 80% confluent 293 cells were grown in 90mm diameter petri dishes and infected at a multiplicity of 0.1 pfu/cell of crude seed stock. Virus was absorbed to the cell monolayer in 0.3ml Tris/saline for 1-1.5hr at 37°C. 15ml of medium containing 1% foetal calf serum (normal medium contained 10% foetal calf serum) was added and monolayers incubated at 37°C for 5-7 days until extensive cpe was evident. Cells were harvested by scraping the cells into the medium with a rubber policeman and decanted into a 50ml Falcon tube. Cells were pelleted by centrifugation at 2,000 rpm for 10min at 4°C in a Beckman GPR centrifuge. The supernatant was aspirated and discarded. The pellet was resuspended in 2ml of Tris/saline, transferred to two black cap vials and frozen and thawed three times. The contents of the vials were

recombined in a 15ml Falcon tube and recentrifuged as above. The supernatant was stored in small aliquots at -70° C.

3.2.2.2. Titration of an Adenovirus Stock.

Serial 10-fold dilutions of virus were made in Tris/saline and 100 μ l of each dilution was added to a 50mm petri dish containing an 80% confluent monolayer of 293 cells. Virus was absorbed at 37°C with occasional gentle rocking. Monolayers were overlaid with 5ml of Eagles agar overlay medium prewarmed to 37°C and incubated at 37°C for 10 days. On day 4 or 5 monolayers were supplemented with 2ml fresh Eagles agar overlay. On day 10 monolayers were fixed with glutaraldehyde for a minimum of 3hr, then stained with giemsa and the number of pfu/ml calculated.

3.3. The In Vitro Latency System.

The model system outlined below and in Figure 3.1 was described in detail by Russell and Preston (1986).

Usually 35mm plates of confluent HFL cells were infected with 3000 pfu/plate in a 0.3ml volume of EF10. The monolayers were incubated at 37°C for 1hr with occasional rocking to allow the inoculum to adsorb to and penetrate the cells. The inoculum was removed with a pasteur pipette and cultures washed gently with 2ml EF10 twice. Monolayers of infected HFL cells were overlaid with 2ml EF5 prewarmed to 42°C and incubated at 42°C for 6 days. On the 6th day cultures were shifted to 37°C (the temperature permissive for virus growth) for a further 4 days. Throughout this 10 day period medium was replaced with 2ml of EF5 (prewarmed to the appropriate temperature) on alternate days. After 4 days at 37°C monolayers were examined for spontaneous reactivation by visual inspection. Monolayers free from spontaneous reactivation were superinfected with a relevant virus over a range of moi (usually 0.1, 0.01, 0.001 pfu/cell) or mock-superinfected. The superinfecting virus was permitted to adsorb to and penetrate the latently infected HFL cells for 1hr at 37°C. For examination of plaque numbers monolayers were overlaid directly with EHu2

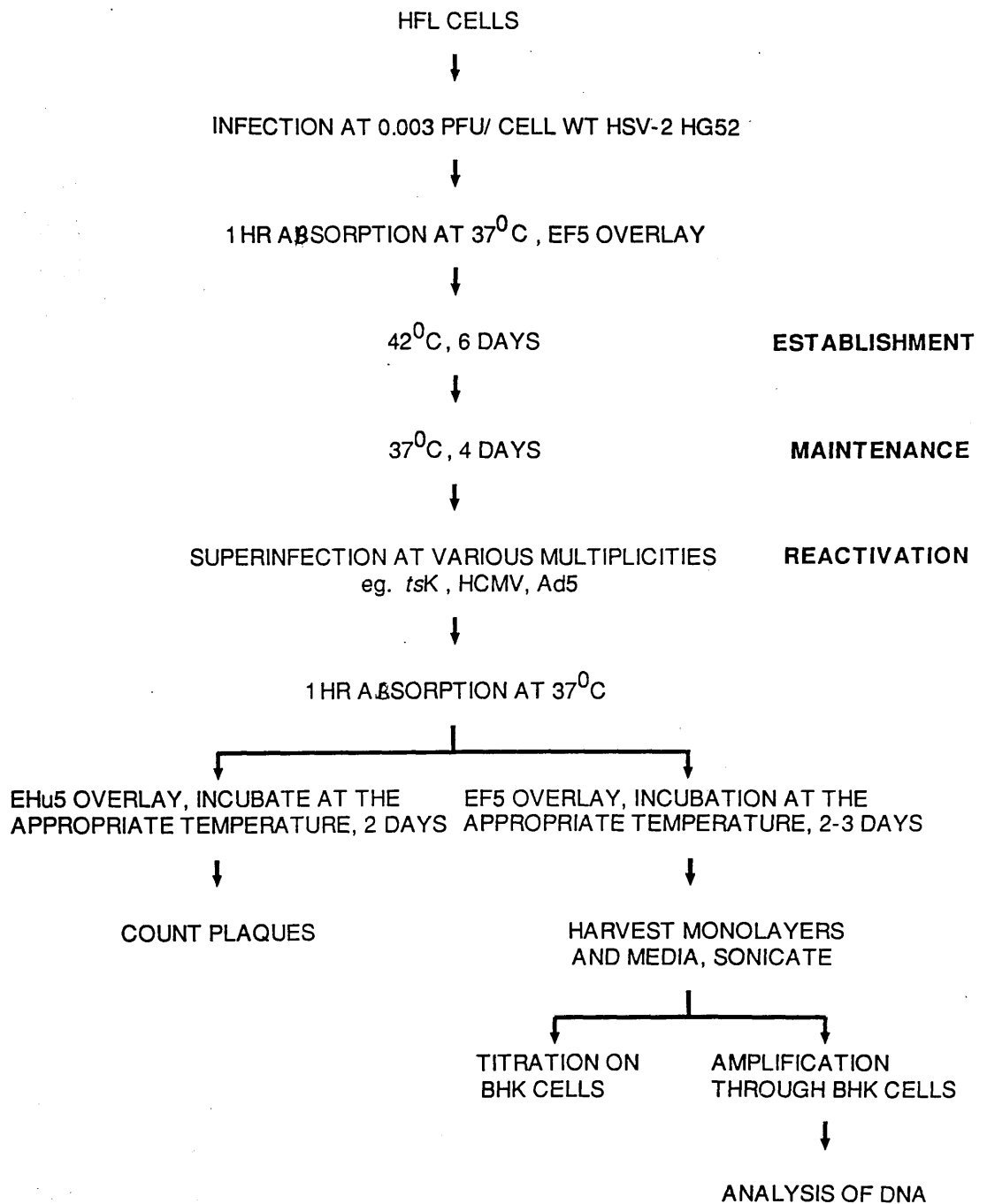


Figure 3.1. SCHEMATIC REPRESENTATION OF THE IN VITRO MODEL FOR HSV LATENCY

or EHu5, incubated at the appropriate temperature for 2-3 days and stained with giemsa. Alternatively, monolayers were overlaid with 2ml EF5 for 2-3 days until cpe appeared, then the cells and medium were combined, sonicated and titrated on or reamplified (for cytoplasmic DNA extraction) by infection of a BHK cell monolayer.

The system was modified slightly to allow the establishment of a high moi stable latent infection of HFL cells with in1814. Routinely at 37°C (with or without prior 42°C treatment) monolayers latently infected with in1814 were overlaid with 2ml EF5 plus 50µg/ml Ara-C.

3.4. Large Scale Preparation of Plasmid DNA.

Routine large scale preparations of plasmid DNA were carried out using a modified "hard-lysis" procedure (Guerry et al., 1973).

50µl of the glycerol stock of bacteria containing the required plasmid was added to 10ml L-Broth plus 50µg/ml ampicillin and incubated at 37°C overnight. From this overnight culture 2ml was used to inoculate 200ml L-Broth plus antibiotic and shaken at 37°C for 6hr. Chloramphenicol was added to a final concentration of 25µg/ml and incubation continued overnight. The culture was centrifuged at 8,000 rpm in a Sorvall GSA rotor for 10min. The supernatant was decanted and the pellet resuspended in 5ml 25% sucrose/50mM Tris-HCl, pH8.0. 2.5ml of freshly made 10mg/ml lysozyme was added and the mixture put on ice for 30min. 2ml of 250mM EDTA, pH7.5 was then added and incubation continued on ice for a further 5min, after which 1.5ml 5M NaCl and 1.5ml 20% SDS were each added in turn. The mixture was mixed well and left on ice for 2-3hr and then centrifuged at 20,000 rpm in a Sorvall SS34 rotor. The supernatant was extracted twice with phenol/chloroform and once with chloroform followed by precipitation in 2 volumes of ethanol at -20°C. The partially purified DNA was pelleted by centrifugation at 2,500 rpm for 15min in a Beckman GPR centrifuge and dissolved in 9ml 20mM Tris-HCl, pH7.5, 100mM NaCl, 1mM EDTA plus 20µl 5mg/ml RNase and incubated at 37°C for 2-4hr. 0.5ml of a 100µg/ml proteinase K solution was added and

incubation continued at 37°C overnight. The proteinase K was removed by two phenol/chloroform extractions and a chloroform extraction. The final volume was adjusted to 9ml with water, and 1ml 3M sodium acetate, (pH7.0) was added. The DNA was precipitated at room temperature by addition of 5ml of isopropanol. After centrifugation at 5,000 rpm for 15min at 20°C in a Beckman GPR centrifuge the pellet was washed with ethanol, dried and dissolved in 300µl distilled water (DW).

3.5. Extraction of Nucleic Acid.

3.5.1. Preparation of Cytoplasmic and Nuclear DNA.

Monolayers on 35mm petri dishes were harvested for cytoplasmic DNA extraction when cpe was evident. The medium was removed carefully and the cell monolayers washed with 2ml of ice cold PBS. 1ml of fresh PBS was added and cells were scraped into this using a rubber policeman. The cell suspension was transferred to 1.5ml reaction vials and centrifuged for 3min at 5,000 rpm in a benchtop microfuge. The supernatant was decanted and the cell pellet resuspended in 100µl TK lysis buffer by vortexing. Samples were placed on ice for 5min and then centrifuged at 13,000 rpm for 2min in a benchtop microfuge. The supernatant was transferred to a fresh vial and 300µl of TEN was added. Samples were phenol/chloroform extracted and ethanol precipitated. RNA present in the cytoplasmic fraction was degraded by treatment with 100µg/ml RNase A at 37°C for 3hr. Five monolayers, on 50mm petri dishes, of latently infected HFL cells were scraped into 10ml PBS with a sterile rubber policeman and transferred to a 50ml Falcon tube. Cells were pelleted at 2,000 rpm for 5 min in a Beckman GPR centrifuge. 1.5ml of TK lysis buffer was added to the pellet, and the mixture was vortexed and incubated on ice for 5min. The sample was centrifuged at 2,500 rpm in a Beckman GPR centrifuge to pellet the nuclei, which were resuspended in 9ml CLB plus 0.25mg/ml proteinase K. The mixture was incubated at 37°C overnight. NaCl was added to 0.1M and the glutinous extract extracted with phenol/chloroform. The DNA

precipitated immediately at room temperature in 2 volumes of ethanol and was lifted from the ethanol with a pipette. Dried DNA pellets were resuspended in 1ml of DW.

The volumes of solutions used were adjusted according to the scale of the cell harvest.

3.5.2. Preparation of Total Cell RNA.

Total RNA was isolated by single extraction with an acid GSCN-phenol-chloroform mixture described by Chomczynski and Sacchi (1987). This rapid method provides a pure preparation of undegraded RNA in high yield.

To each monolayer of cells on a 50mm petri dish, 0.9ml GSCN buffer was added and the lysed cell mix transferred to a 10ml Sarstedt polypropylene tube. Sequentially, 90 μ l 2M sodium acetate, pH4.0, 0.9ml phenol (saturated with water), and 180 μ l of chloroform/iso-amyl alcohol mixture (49:1) were added with thorough mixing after addition of each reagent. The final solution was vortexed for 10sec and cooled on ice for 15min. Samples were centrifuged at 9,000 rpm for 15min at 4°C in the outer ring of a Sorvall SM24 rotor. The aqueous phase was transferred to a fresh tube, mixed with 0.9ml of isopropanol and precipitated at -20°C for 1hr exactly. Sedimentation of RNA at 9,000 rpm for 15min in the outer ring of a Sorvall SM24 rotor was performed and the resulting pellet dissolved in 0.3ml GSCN buffer and an equal volume of isopropanol. The sample was precipitated at -20°C for >1hr or overnight. After centrifugation as above the pellet was resuspended in 800 μ l DEP-treated DW, 0.3M sodium acetate and 75% ethanol. The RNA was stored at this stage as a salt and ethanol precipitate at -20°C until ready for use. RNA was sedimented as above, the pellet washed twice with 70% ethanol and dissolved in 50 μ l DEP-treated sterile DW. The concentration of the RNA stock was adjusted to 5 μ g/ μ l.

3.6. Separation of DNA by Gel Electrophoresis.

3.6.1. Minigels.

Small amounts of DNA were analysed by electrophoresis in a 0.8% agarose, 50ml minigel (100mm x 70mm x 7mm) in 1 x TBE. Samples were loaded in 1 x TBE loading buffer in a total volume of 10 μ l and electrophoresed in 1 x TBE at 40mA for 1hr. The gel was stained by addition of 3 μ g/ethidium bromide after 45min, visualised by exposure to UV illumination and photographed on Polaroid 667 film.

3.6.2. Large Agarose Gels.

300ml horizontal slab gels (260mm x 160mm x 7.5mm) containing 1% agarose in 1 x E buffer plus 0.5 μ g/ml ethidium bromide were used for analysis and preparation of DNA restriction fragments. Alternatively, for Southern transfer of DNA samples, a 0.6% agarose, 1 x E buffer, 0.5 μ g/ml ethidium bromide, 200ml horizontal gel (260mm x 160mm x 5mm) was employed. Samples were loaded in 1 x dye-ficoll and electrophoresed in 1 x E buffer (plus 0.5 μ g/ml ethidium bromide) at 40mA for 16-18hr. Separation of high molecular weight latent DNA was achieved by electrophoresis at 50mA for 20hr.

3.6.3. Polyacrylamide Gels.

Vertical non-denaturing gels were used for resolving fragments generated by PCR amplification of HSV specific sequences.

A 50ml solution containing 6% acrylamide (from a stock solution of 29% acrylamide and 1% N,N'methylene bisacrylamide), 0.55 x TBE and 10% ammonium persulphate was prepared. 50 μ l TEMED was added immediately prior to pouring the mixture into a gel sandwich (260mm x 160mm x 1mm). Samples were loaded into the polymerized gel in 1 x TBE loading buffer with a capillary tube. Electrophoresis was carried out in 0.55 x TBE at 200V for 3-4hr. Bands were visualized by soaking the gel in 0.55 x TBE and 1 μ g/ml

ethidium bromide prior to UV illumination. Gels were photographed on Polaroid 655 film and those containing radiolabelled DNA fragments were dried and subjected to autoradiography.

3.7. Purification and Characterization of Nucleic Acids.

3.7.1. Restriction Enzyme Digestion.

DNA was routinely digested in a volume of 20-30 μ l, in reaction conditions specified by the manufacturer of the enzyme, with the addition of 0.01% BSA. Restriction digests were generally carried out at 37°C for 3hr.

Digestion of high molecular weight total cellular DNA was carried out in a 1.5ml volume at 37°C overnight in the presence of 100 units BamH1, an additional 50 units of BamH1 was added and incubation was continued for a further 5hr period.

3.7.2. Phenol/Chloroform Extraction of Nucleic Acids.

Purification of DNA from protein was achieved by extraction in an equal volume of distilled phenol and chloroform mixed in a 1:1 ratio, agitation and centrifugation at 13,000 rpm for 5min in a benchtop microfuge for samples <1.5ml or at 2,000 rpm for 15min in a Beckman GPR centrifuge. The aqueous phase was re-extracted as above and then extracted once with chloroform to remove residual phenol. Generally, DNA in aqueous solution was precipitated by addition of sodium acetate to 0.3M or NaCl to 0.1M in an equal volume of isopropanol at room temperature for >2hr or in 2-3 volumes of ethanol at -20°C for >2hr.

DNA was pelleted by centrifugation at 13,000 rpm in a benchtop microfuge for 10min or 2,500 rpm for 15min in a Beckman GPR centrifuge. DNA was then washed in ethanol, dried and dissolved in DW.

RNA was pelleted by centrifugation at 9,000 rpm in the outer ring of a Sorvall SM24 rotor, washed with 70% ethanol, dried and dissolved in sterilized DEP-treated DW.

3.7.3. Ultracentrifugation of DNA in a Caesium Chloride Gradient.

Purification of viral DNA from latently infected cells was achieved by ultracentrifugation of DNA through a gradient of refractive index (RI) 1.4 (density 1.707g/ml). The basis of this protocol was the differential high G+C content of HSV DNA sequences relative to the majority of cellular sequences. HSV infected cellular DNA isolated from five 50mm plates was digested with BamH1 to completion and mixed with a solution of CsCl to a final RI of 1.4 and a volume close to 18ml. The mixture was transferred to a TV865B centrifuge tube, balanced with CsCl of RI 1.4 and filled to the base of the tube neck with liquid paraffin. The tube was sealed in a crimper and centrifuged in a TV865B vertical rotor at 40,000 rpm in a Sorvall OTD50B ultracentrifuge at 15°C for approximately 18hr. The gradient was collected in 10 drop fractions by piercing the base of the centrifuge tube with a syringe needle. The RIs of the samples were determined and those with a RI between 1.4036 and 1.4002 were pooled with 20µg E.coli RNA and dialysed against 1 x TE for 2-3hr at 4°C. Dialysis buffer was changed and the dialysis continued overnight. The volume of the sample was reduced to approximately 1ml by addition of an equal volume of butan-2-ol. The upper phase was discarded and remaining butan-2-ol evaporated by incubation at 65°C for 2hr. The final sample was divided into 2 parts of 0.5ml each and precipitated in ethanol. The washed and dried pellet was dissolved in 40µl DW.

3.7.4. Electroelution of DNA Fragments.

The required DNA fragment excised from an agarose gel was eluted onto dialysis membrane in 1 x EEB in an electroelution chamber at 20mA/sample at 4°C for 1.5hr. The DNA was removed from the dialysis membrane in 200µl 1 x EEB by vigorous pipetting. The final volume of the sample was adjusted to 400µl by addition of sodium acetate, pH7.0, to 0.3M and DW. The preparation was extracted with phenol/chloroform, isopropanol precipitated, dried and

redissolved in DW.

3.7.5. Estimation of Nucleic Acid Concentration.

A series of dilutions of plasmid DNA, linearized with an appropriate restriction endonuclease, or of a purified DNA fragment were prepared and electrophoresed in a minigel beside an applicable DNA control of known concentration. DNA bands were visualized under UV illumination and photographed on Polaroid 665 film. An estimate of DNA concentration was derived by densitometric analysis of images on negative film.

Estimations of HSV DNA concentrations in in vitro latency experiments (Section 4.4.2.) were obtained by densitometric analysis of autoradiographs using a Joyce-Loebel scanning densitometer. The areas beneath the peaks of the densitometric trace were determined utilizing a programme, DENS, written by Dr. P. Taylor for a DEC PDP 11/44 computer linked to a digitizing table.

The concentration of total cell RNA was estimated by measuring the OD at 260nm and 280nm in a Beckman Du-62 spectrophotometer.

3.8. In Vitro ³²P-Labeling of DNA Fragments.

3.8.1. Nick Translation.

DNA was labelled with ³²P as described by Rigby et al. (1977). 0.5μg of plasmid DNA in 2.5μl of 1 x Nick translation buffer and 20μm each of non-radioactive dATP and dTTP was incubated at room temperature for 2-3min following addition of 2x10⁻⁴μg DNase. The mixture was placed on ice and DW added so that the final volume after addition of radioactive isotopes and enzyme was 25μl. 3μl (30μCi) each of α-[³²P]-dCTP and α-[³²P]-dGTP were added followed by 2 units of E.coli DNA polymerase I. The reaction was incubated at 14°C for 1hr and stopped by addition of 4μl 250mM EDTA, pH7.5. Unincorporated dNTPs were separated from ³²P-labelled DNA by centrifugation through a 1ml Sephadex G50 (fine) spun column at 2,000 rpm in a Beckman GPR

centrifuge.

3.8.2. Hexanucleotide Primer Extension.

Random primer extension was carried out as described by Feinberg and Vogelstein (1983). 100ng of DNA in 20 μ l was heated in a dri-block at 90°C for 10min, centrifuged briefly and cooled on ice. The DNA was incubated in a 50 μ l reaction mix containing 0.4mg/ml BSA, 10 μ l oligo reaction mix, 20 μ M each non-radioactive dATP, dGTP, dTTP, 5 μ l (50 μ Ci) of α -[³²P]-dCTP and 6 units of Klenow fragment DNA polymerase at room temperature overnight. The labelled DNA fragment was purified through a G50 sephadex spun column.

3.8.3. 5'-end Labelling of Fragments.

15-20mmoles of the 5' (sense) oligonucleotide in 1 μ l was incubated at 37°C for 1-2hr in kinase buffer plus 50 μ Ci of γ -[³²P]-ATP and 2 units of T₄ polynucleotide kinase in a total volume of 50 μ l (Arrigo *et al.*, 1989). The labelled probe was purified by passage through a 10ml Sephadex G50 (medium) column. The probe was eluted in approximately 1ml of DW.

3.8.4. Determination of Percentage Incorporation.

A 3 μ l volume of a 1/10 dilution of a sample from the labelling reaction was spotted onto a Whatman No.1 filter paper disc. The discs were dried under a heat lamp and washed once in 10% (w/v) trichloroacetic acid (TCA), once in 5% (w/v) TCA and twice in ethanol. Discs were redried, placed in a scintillation vial containing 3ml Ecoscint A (National Diagnostics). Radioactivity was measured in a scintillation counter and compared to an equivalent sample that had not been TCA washed (total counts).

3.9. Southern Transfer and Hybridization.

The method employed was essentially as described by Southern (1975) with modifications as indicated.

3.9.1. Transfer of DNA from Agarose to a Membrane.

Following electrophoresis DNA fragments were transferred to GeneScreen Plus membrane (DuPont) by alkaline transfer (Chomczynski and Qasba, 1984). The agarose gel was placed in transfer solution for 30min with gentle agitation. The gel was then laid on two sheets of pre-wet Whatman 3MM paper supported by a glass plate and designed to act as a wick. GeneScreen Plus membrane, cut to dimensions identical to the gel, was pre-soaked in transfer solution and placed "B" side down on the gel. Capillary transfer in transfer solution was achieved through two sheets of 3MM paper and a large stack of paper towels. A weight was placed on the towels and DNA transfer allowed to proceed for 16-18hr. The blotting assembly was dismantled and the membrane soaked in neutralizing solution for 15min with occasional shaking. The membrane was dried at room temperature for a minimum of 1hr.

3.9.2. DNA/DNA Hybridization.

The GeneScreen Plus membrane containing immobilized DNA fragments was incubated at 65°C for 2hr in 100ml of prehybridization solution sealed in a polythene bag in a shaking water bath. The radiolabelled probe in 800µl DW was denatured by addition of 200µl 1M NaOH for 10min at room temperature. The solution was neutralized by addition to 8.8ml hybridization solution plus 200µl 1M HCl. The prehybridization solution was removed from the polythene bag and replaced with 10ml of hybridization solution containing the ³²P-labelled DNA probe. Hybridization was carried out at 65°C for 16-18hr in a shaking water bath. The hybridization solution was removed and the membrane washed at room temperature for 45min in 10 x Denhardt's and 1 x SSC, once each at 65°C for 30min in a solution of 2 x SSC and 0.25% SDS, followed by 0.3 x SSC and 0.1% SDS. Finally, the membrane was washed in water and allowed to air dry before autoradiography.

3.10. Purification of Oligonucleotides.

The supplied oligonucleotide, in a volume of approximately 1ml, was heated at 55°C for 5hr to remove protecting groups and dispensed into four equivalent samples. Two portions were stored at -70°C, the remaining oligonucleotide samples were lyophilised and redissolved in 50µl 90% (v/v) deionised formamide and 1 x TBE. A 150ml denaturing acrylamide gel (15% acrylamide with 4% crosslink) was polymerised for 1hr and pre-run in 1 x TBE for 2hr at 20W. Urea was flushed from the wells with a 50ml syringe and 10µl of xylene cyanol/bromophenol blue loaded in an end well and run in parallel with the oligonucleotide samples. Electrophoresis was carried out at 35W for about 3hr or until the marker dye band had progressed beyond 7cm. The oligonucleotide bands were visualized by the shadow casting technique. In this method, the gel was removed from the gel plates onto clingfilm and placed on a fluorescent thin layer chromatography plate under a shortwave UV lamp. The slowest moving dark band was excised with a brand new scalpel blade, diced and incubated overnight at 45°C in 2ml gel elution buffer. Oligonucleotide samples were separated from acrylamide by passing the mixture through a Whatman glass microfibre GF/C filter. The filtrate was dispensed in 0.4ml aliquots and 2 volumes of ethanol added to precipitate samples at -20°C. Oligonucleotides were centrifuged at 15,000 rpm in a Sorvall SS34 rotor, washed in ethanol, dried and resuspended in 50µl DW.

3.11. Amplification of HSV Specific ^{RNA} Sequences by PCR.

The technique utilised was a modification of that described by Saiki et al. (1988). Autoclaved solutions (where possible) that had been divided into small aliquots and positive displacement pipettes were used throughout to minimise the possibility of contamination.

50µg of RNA was treated with 1 unit of RNase free DNase in a 50µl volume of DNase buffer at 37°C for 1hr. The samples were extracted with water saturated phenol/chloroform-isoamyl alcohol and precipitated in 2

volumes of ethanol at -20°C for 2hr. Precipitated RNA was centrifuged for 10min at 13,000 rpm in a benchtop microfuge, washed, dried and dissolved in $10\mu\text{l}$ of 250mM KCl. RNA samples were boiled at 100°C for 1min in a 0.5ml reaction vial in a $11\mu\text{l}$ volume that contained approximately $50\mu\text{g}$ RNA and 0.5-0.6mmoles antisense oligonucleotide. Vials were cooled on ice. The RNA/primer mixture was subjected to a primer extension hybridization reaction and incubated at the hybridization temperature for 30min in 250mM KCl. The hybridization temperature is determined by the number of each nucleotide in the primer sequence, and thus is unique to each primer and given by the equation :

$$4(\text{G}+\text{C}) + 2(\text{A}+\text{T}) - 5^{\circ}\text{C}$$

Samples were cooled and conditions adjusted by the addition of 1 x reaction buffer, 40 units of MMLV reverse transcriptase and DW to a total reaction volume of $50\mu\text{l}$. RNA samples were converted to complementary DNA by reverse transcription at 42°C for 1hr. Complementary DNA samples were phenol/chloroform-isoamyl alcohol extracted, NaCl was added to 0.1M and precipitated in an equal volume of isopropanol at room temperature for 2hr. Centrifugation was carried out at 13,000 rpm in a benchtop microfuge for 10min and pellets dissolved in $20\mu\text{l}$ DW. A $5\mu\text{l}$ aliquot of primer extended RNA was subjected to PCR. The PCR amplification mix contained 1 x PCR buffer, $200\mu\text{M}$ each dATP, dTTP, dCTP, 0.5-0.6mM unlabelled antisense oligonucleotide, 0.5-0.6mmoles sense oligonucleotide 5'-end labelled with T_4 polynucleotide kinase (Arrigo *et al.*, 1989) and 1 unit of Tag polymerase. Each $50\mu\text{l}$ reaction was supplemented with the following :

LAT specific reactions : $150\mu\text{M}$ dGTP and $50\mu\text{M}$ 7-deaza-2' deoxyguanosine.

TK specific reactions : $200\mu\text{M}$ dGTP.

Each PCR amplification was performed under $100\mu\text{l}$ of mineral oil in an automated thermal cycler (Cambio) as follows : 1 cycle of denaturation at 93°C for 6min, annealing at 55°C

for 0.1min and polymerization at 72°C for 4min. 30 cycles of 93°C for 1min, 55°C for 0.1min, 72°C for 4min and completed with 2 cycles at 93°C for 0.1min, 55°C for 0.1min, and 72°C for 8min. PCR amplified products (10 μ l) were separated on a 6% polyacrylamide non-denaturing gel, and the gel was dried and the bands visualised by autoradiography.

3.12. Analysis of Polypeptides from Virus Infected Cells.

3.12.1. Pulse-Labeling of Cells.

Confluent monolayers of HFL cells in 35mm petri dishes were mock infected or infected with 5 particles/cell of in1814 or 1814R. Following incubation at 37°C for 1hr the monolayers were overlaid with 2ml EF5 and incubated at the appropriate temperature (42°C or 37°C). At a predesignated time post-infection cultures were pulse-labelled. HFL monolayers were washed with prewarmed PBS and 0.3ml of 50 μ Ci/ml [³⁵S]-methionine and actinomycinD(1 μ g/ml) in PBS was added. After 1hr the radiolabelled medium was decanted and the cells were washed once with ice cold PBS followed by addition of 0.2ml ice cold 1 x TE. The cells were harvested after 5-10min on ice with a rubber policeman. The labelled cell mixture was transferred to a 1.5ml reaction vial, mixed with 100 μ l of 3 x boiling mix and heated to 90°C for 15min. At this stage samples were stored at -70°C until analysis.

3.12.2. SDS-Polyacrylamide Gel Electrophoresis.

A gel mix solution was prepared by combining 10ml of gel buffer, 13ml 30% acrylamide/3% DATD, 0.5ml 10% (w/v) ammonium persulphate in a 40ml volume, 40 μ l TEMED was added last and mixed. The gel mix was poured into a prepared gel sandwich (260mm x 160mm x 1mm) to fill 75% of the space. Using a pasteur pipette, 20ml of 0.25 x gel buffer was used to overlay the gel mix immediately to ensure a good interface. After about 1hr when the acrylamide had polymerized the 0.25 x gel buffer was poured off. 20ml of SGB [plus 0.5ml 10% (w/v) ammonium persulphate and 25 μ l TEMED] was added after the surface of the gel had been

rinsed twice with 0.25 x gel buffer. 15min later the well former was removed and excess acrylamide displaced by rinsing the wells with tank buffer. Samples were electrophoresed in a vertical tank at 40mA constant current for approximately 5hr in tank buffer. The gel was soaked overnight in a 1:1 mix of gelfix and destain at room temperature, dried under vacuum and autoradiographed.

3.13. Estimation of Protein Concentration.

An estimation of protein concentration was made by the method described by Bradford (1976). 10 μ l of cell extract was added to 1ml of Bradford reagent (0.01% comassie brilliant blue G, 0.0003% SDS, 4.75% (v/v) ethanol, 8.5% phosphoric acid) in plastic curvettes. Samples were covered with a portion of Nesco film, inverted and left for 10min at room temperature. The absorbance at 595nm was measured and compared with the standard curve of known concentrations of BSA at OD₅₉₅.

3.14. Determination of Thymidine Kinase Activity.

Cells in 35mm petri dishes were infected at the appropriate moi, incubated at 37°C or 42°C and cytoplasmic extracts were made as described in Section 3.5.1 with the exception that 6.5mM β -mercaptoethanol was incorporated in the TK lysis buffer. Up to 20 μ l of cytoplasmic cell extract was assayed in a reaction mix of 50 μ l containing 10mM MgCl₂, 5mM ATP, 100 μ M dTTP and 100 μ Ci/ml [³H]-thymidine (47Ci/mmol) at 30°C for 2hr. The reaction was terminated by addition of 10 μ l of a 1:1 mix of 2mM thymidine and 50mM EDTA, pH7.5 and heated in a dri-block to 90°C for 3-4min. Samples were cooled on ice for 5min and then centrifuged in a benchtop microfuge at 13,000 rpm for 5min. 50 μ l of the supernatant was spotted onto Whatman DE81 discs which, after drying, were then washed 3 times in 4mM ammonium formate (pH4.0), 10mM thymidine at 37°C and a further 2 times in absolute alcohol at room temperature for 5min each. Discs were dried under a heat lamp and placed in scintillation vials plus 3ml Ecoscint A (National Diagnostics). Radioactive counts per

minute (cpm) were measured in a scintillation counter.

4.1. RESULTS.

4.1. Objectives.

A distinctive feature of HSV is the ability to reside in the neuronal cells of the sensory ganglia in a latent state and to persist in this non-infectious form throughout the lifetime of its host. The aim of the work presented in this thesis was to investigate, at the molecular level, the events which occur during establishment and maintenance of the latent state, and during reactivation from latency using, a well characterized tissue culture latency system that is schematically outlined in Figure 3.1. (Russell and Preston, 1986; Russell et al., 1987; Russell, 1989). The in vitro latency system relies on the ability of HFL cells to withstand prolonged periods of incubation at the supraoptimal temperature of 42°C. Infection of HFL cell monolayers at low moi results in a virus-cell interaction that is stable after incubation at 42°C for 6 days followed by 4 days at temperature permissive for virus growth. The latent virus genome is activated by superinfection of the cultures, for example with tsK at 38.5°C, by which means 30-100% of the initial inoculum is recovered.

Emphasis was placed on the examination of the reactivation process, particularly the role of the HSV-1 polypeptide Vmw110, and characterization of mutant in1814 to elucidate the physical organization of the latent viral genome and the status of viral gene expression. Recent evidence has indicated that during latency in vivo, the HSV genome is probably a circular episome that is in a chromatin-like arrangement (Rock and Fraser, 1983,1985; Efsthathiou et al., 1986; Mellerick and Fraser, 1987; Deshmane and Fraser, 1989) and transcription is limited to a family of RNA molecules originating from the long repeats of the genome (Croen et al., 1987; Rock et al., 1987a; Spivack and Fraser, 1987; Stevens et al., 1987).

4.2. Experimental Reactivation Induced by Superinfection.

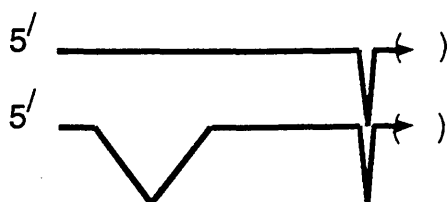
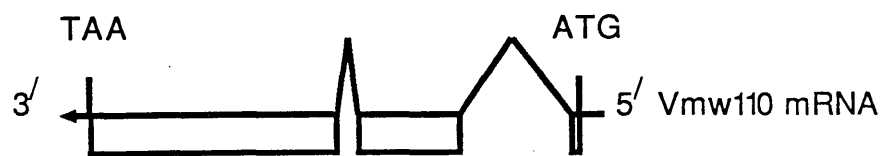
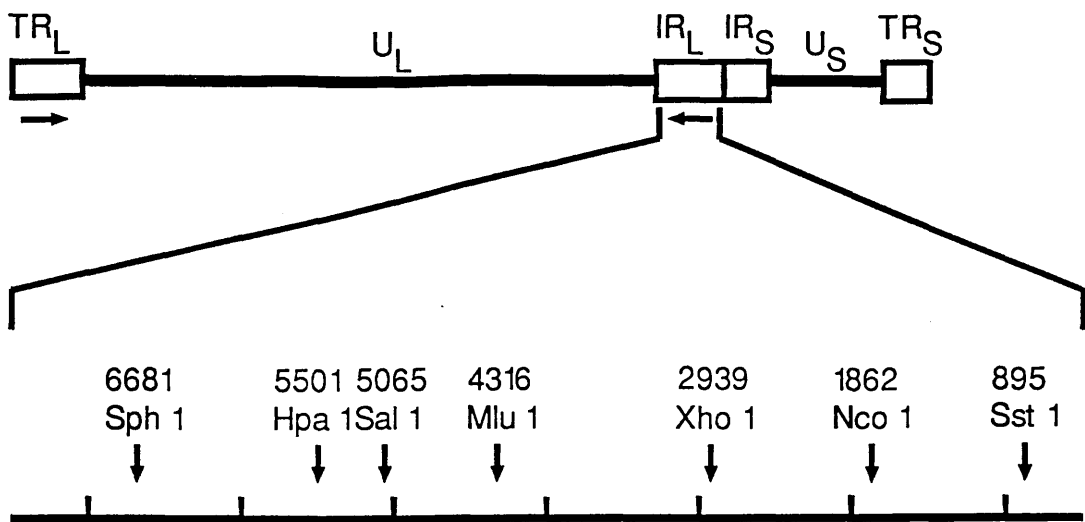
Previous findings utilizing the in vitro latency system demonstrated that latent HSV-2 could be reactivated experimentally at high efficiency by superinfection of monolayers with wt HSV-1 or HCMV but not with Ad5 (Russell et al., 1987). Reactivation of the latent HSV-2 genome by intertypic superinfection with ts mutants that do not synthesise functional Vmw175 and are defective for progression to later phases of HSV gene expression demonstrated that at most only the IE proteins plus the early polypeptide Vmw136 were involved in reactivation (Russell et al., 1987). Interestingly, mutant dl1403 failed to rescue latent HSV-2 upon superinfection of latently infected cultures, implying a role for Vmw110 in the process of reactivation. Dl1403 is deleted for 2kbp in both repeats bounding U_L such that the altered polypeptide it is predicted to encode is derived from the N-terminal 105 amino acids (exon 1 and part of exon 2) fused to 56 residues specified by an ORF that is not normally used (Stow and Stow, 1986, Figure 4.1.). Recent characterization of the transcripts produced during latency revealed that dl1403 was, in fact, a double mutant as the majority of the 3' portion of LAT which overlaps Vmw110 is also removed (Figure 4.1.). Therefore, the lack of reactivation induced by superinfection with dl1403 did not exclude the possibility of a role for LAT. Furthermore, in infected BHK cells the profile of proteins synthesised by dl1403 are indistinguishable from those produced by wt HSV-1 apart from the absence of Vmw110 (Stow and Stow, 1986). Therefore, other HSV polypeptides, particularly IE proteins, may act in conjunction with Vmw110 during the reactivation process. These aspects of reactivation were investigated using adenovirus recombinants and defined HSV-1 deletion mutants.

4.2.1. Reactivation Induced by Superinfection with Adenovirus Recombinants.

The Vmw110 genomic coding sequence, controlled by the natural Vmw110 promoter or the adenovirus major late

Figure 4.1. POSITIONS OF THE DELETION MUTANTS D11403, FXE AND D14 IN RELATION TO LAT AND THE Vmw110 TRANSCRIPT.

Numbering of nucleotides begins at the IR_L junction a sequence (Perry et al., 1986). The genomic locations of Vmw110 and LAT in IR_L are indicated in the expansion; the exact 3' end of LAT is uncertain as denoted by the parentheses. The dashed line represents the 2.0kbp region deleted in d11403. Solid boxes indicate the position of deletions D14 and FXE.



LAT



dl 1403

D14

FXE

promoter, was cloned into the the E1 region of Ad5 generating Ad110-110 and AdMLP-110 respectively (Zhu et al., 1988). Upon infection of HeLa or 293 cells, the hybrid adenoviruses expressed a Vmw110 transcript and polypeptide at late times that appeared identical to that produced by wt HSV-1. Virus replication in the recombinant infected cells occurred only in 293 cells which supply E1a gene products in trans, indicating that Vmw110 was unable to substitute for E1a functions (Zhu et al., 1988).

HFL cell monolayers were mock infected or infected with HSV-2 at 0.003 pfu/cell and, following absorption at 37°C, the cultures were maintained at 42°C for 6 days and then at 37°C for a further 4 days. The viruses tested for the ability to reactivate latent HSV-2 in culture were HCMV, tsKsyn, Ad5, Ad5 dlE1,3 (Haj-Ahmad and Graham, 1986) and the adenovirus recombinants. Cultures were superinfected at a multiplicity of 0.1 pfu/cell with the various viruses at 37°C, or 38.5°C for tsKsyn. The cultures were harvested and sonicated in growth medium after 3 days at the appropriate temperature when extensive and equivalent cpe was evident. Cell lysates were titrated on BHK monolayers in the presence of human serum and plaque numbers determined after incubation at 37°C or 38.5°C.

It has been demonstrated previously that reactivation of HSV-2 from its latent state can be induced efficiently by superinfection with HSV-1 ts mutant viruses or HCMV in related in vitro latency systems (Colberg-Poley et al., 1979b, 1981; Russell and Preston, 1986). In agreement with these findings, the results presented in Table 4.1. indicate that superinfection at 0.1 pfu/cell with HCMV or tsKsyn rescued latent HSV-2. Similarly, superinfection with the adenovirus hybrids AdMLP-110 or Ad110-110 reactivated latent HSV-2. However, the yield of HSV-2 recovered was consistently lower than that observed upon superinfection with HCMV or tsKsyn possibly because significant amounts of Vmw110 are not produced until late in infection (Zhu et al., 1988). In contrast, the synthesis of Vmw110 in tsKsyn infected cells occurs during the IE phase of infection. Furthermore, at the NPT tsKsyn IE gene products accumulate to high levels (Preston, 1979b). In comparison,

Table 4.1. REACTIVATION OF LATENT HSV-2.

SUPERINFECTING VIRUS ^a	NUMBER OF REACTIVATED CULTURES ^b	MEAN TITRE (pfu/ml) ^c
Mock	0	-
Ad5	1	1.0×10^2
Ad5 <u>dlE1</u> ,3	0	-
<u>tsKsyn</u>	5	1.1×10^5
HCMV	5	1.1×10^5
AdMLP-110	5	7.6×10^3
Ad110-110	5	2.8×10^3

a HFL cells were infected with 0.003 pfu/cell of HSV-2 and superinfected with the indicated virus at 0.1 pfu/cell following incubation at 42°C for 6 days and 4 days at 37°C. Superinfection with tsKsyn was carried out at the NPT 38.5°C.

b In each case the number of cultures examined for reactivation was 5.

c Superinfected HFL cell cultures and growth medium were harvested, sonicated and titrated on BHK cells.

superinfection of latently infected monolayers with Ad5 dlE1,3 failed to reactivate HSV-2 (Table 4.1.). Ad5 dlE1,3 was included as a control as both hybrids are deleted for the E1 region and AdMLP-110 is also deleted for E3 (Zhu et al., 1988). The majority of latently infected cultures did not release HSV-2 upon superinfection with Ad5 (Table 4.1.). The virus recovered by Ad5 superinfection arose from 1 out of 5 plates and was probably a consequence of a low level reactivation event that has been documented previously (Russell and Preston, 1986), although the possibility that the products of Ad5 E1a can reactivate latent HSV-2 at a reduced frequency, that was not detected in earlier studies, cannot be excluded.

The virus produced following superinfection and culture at the appropriate temperature was identified as HSV-2 by the ability to form plaques on BHK cells, which are non-permissive for HCMV and adenovirus, and the predominance of plaques on this cell type with non-syncytial morphology at the NPT for growth of tsKsyn.

The results presented in this section demonstrate that a functional Vmw110 supplied by the adenovirus recombinants can reactivate latent HSV-2 in the absence of other known HSV proteins.

4.2.2. Superinfection of Latently Infected Cultures with Deletion Mutants of HSV-1.

HSV-1 mutants with defined alterations have been used to examine the specific functional domains of Vmw110 that contribute to the role of this polypeptide in reactivation. Everett (1989) described the construction of a number of HSV-1 mutants with small inframe insertions or deletions within Vmw110. Mutant FXE has a 45 amino acid inframe deletion in the second exon of Vmw110 between nucleotides 2944-3076 with respect to the numbering system described by Perry et al. (1986) (Everett, 1988a, 1989; Figure 4.1.). The deletion in FXE specifically disrupts a cysteine-rich region homologous to the metal ion binding domain described by Berg (1986), although the affinity of Vmw110 for zinc ions is not dependent on the integrity of the cysteine-rich

motif (R.D. Everett, unpublished observations). D14 has a 41 amino acid inframe deletion towards the carboxy end of Vmw110 between bases 4803-4922 (Everett, 1988a,1989; Figure 4.1.). By virtue of the 3' complementarity of Vmw110 and LAT, D14 also lacks 41 amino acids from the C-terminus of the LAT ORF1 and therefore affects LAT whereas FXE does not. Importantly, the domain in which D14 is located is required for transactivation of a heterologous gene in transient transfection assays in the presence, but not the absence, of Vmw175 whereas the region of the polypeptide in the proximity of deletion FXE is crucial for the intrinsic trans-acting function of Vmw110 (Everett, 1988a). Mutant FXE is as impaired for growth on BHK and HFL cells as the larger deletion mutant dl1403 (Everett, 1989). In comparison, D14 exhibits a reduced growth phenotype on these cells that is intermediate between dl1403 and wt HSV-1 (Everett, 1989).

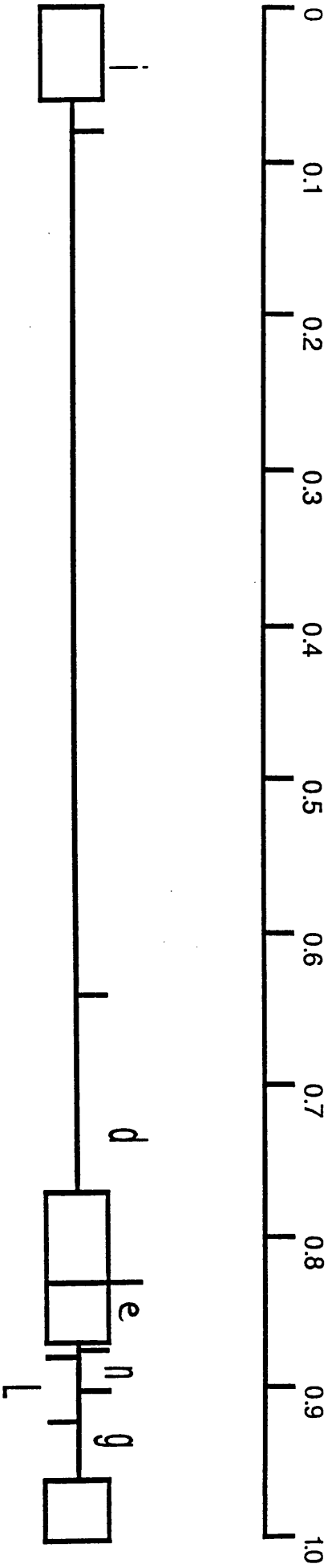
HFL cells infected with 0.003 pfu/cell HSV-2 and maintained at 42°C for 6 days followed by incubation at 37°C for 4 days were superinfected over a range of multiplicities with wt HSV-1, dl1403, FXE and D14, or mock superinfected. Cultures were harvested after 1-2 days at 37°C when extensive cpe was observed. A portion of the cell lysate was amplified by replication through BHK cells, cytoplasmic DNA extracted and digested with HindIII. The DNA fragments were separated by gel electrophoresis and the presence of HSV-2 in these samples was determined by Southern hybridization analysis using a cloned HSV-2 HindIII L fragment (Figure 4.2.) or a cloned 655bp fragment derived from the unique 3' end of the HSV-2 glycoprotein G gene (McGeoch et al., 1987).

In Figure 4.3. the presence of the HSV-2 specific HindIII L fragment in latently infected cultures was distinguished from the HSV-1 bands HindIII b, e, g and n (Figure 4.2.), which cross hybridize, by virtue of the differences in their electrophoretic mobility. The HSV-1 homologous bands act as an internal control indicating the replication efficiency of the superinfecting virus.

As expected, wt HSV-1 reactivated latent HSV-2 over a 100-fold range of superinfecting multiplicities but the

Figure 4.2. THE LOCATIONS OF THE HSV-2 HindIII L AND HOMOLOGOUS HSV-1 HindIII FRAGMENTS ON THE HSV GENOME.

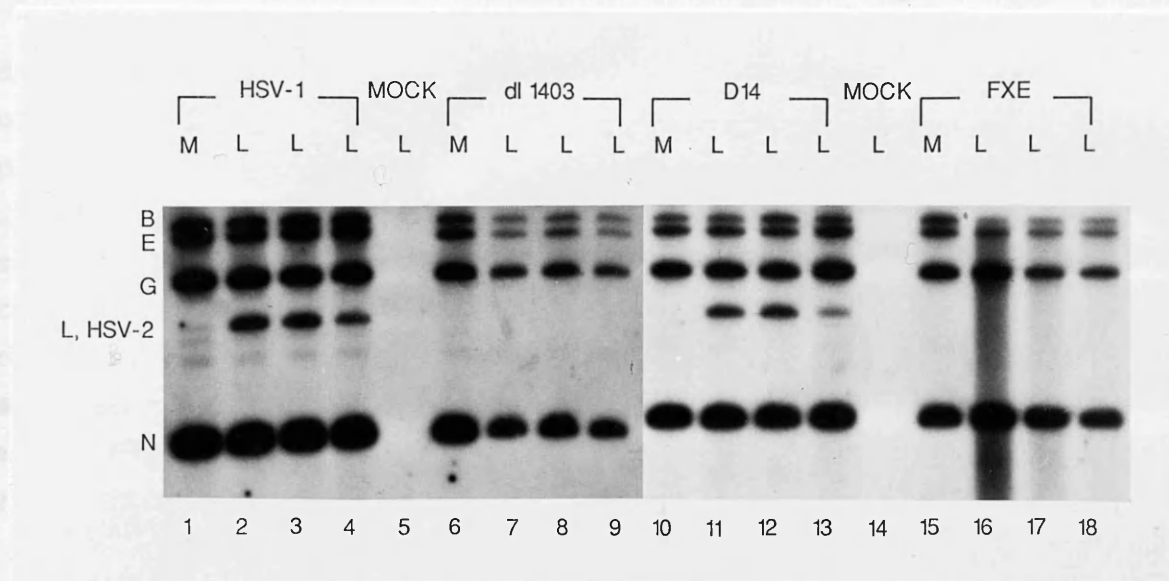
The relative positions of the HSV-2 HindIII L restriction fragment and the HSV-1 fragments HindIII b, e, g and n, which cross hybridize, are illustrated. The scale in μ is indicated above the diagram.



$g+d=b$
 $g+i=e$

Figure 4.3. REACTIVATION OF LATENT HSV-2.

HFL cells were infected with 0.003 pfu/cell wt HSV-2 (lanes labelled L) or were mock infected (lanes labelled M). Cultures were superinfected with 0.1 (lanes 2, 7, 11 and 16), 0.01 (lanes 3, 8, 12 and 17) or 0.001 (lanes 4, 9, 13, and 18) pfu/cell of wt HSV-1 (lanes 1-4), dl1403 (lanes 6-9), D14 (lanes 10-13), or FXE (lanes 15-18). Lanes 5 and 14 contain latently infected samples mock superinfected. Cytoplasmic DNA was extracted from BHK cell monolayers in which reactivated virus progeny was amplified. HindIII restricted DNA was subjected to Southern hybridization analysis using a HSV-2 HindIII L probe.



HindIII L fragment was not detected in cultures that had been superinfected with dl1403. Mutant D14 reactivated latent HSV-2, as shown by the presence of the HindIII L band, whereas FXE did not, although hybridization with HSV-1 specific bands confirmed that FXE, like the other superinfecting viruses, had replicated efficiently.

Hybridization was repeated with a HSV-2 specific probe with which minimal hybridization to HSV-1 sequences was observed (Figure 4.4.). In this experiment, HSV-2 HindIII L was detected in cytoplasmic DNA samples from cultures superinfected with wt HSV-1 or D14 but not dl1403 or FXE. thus corroborating the evidence presented in Figure 4.3.

These results demonstrate that the region of Vmw110 defined by the deletion in FXE is essential for reactivation of latent HSV-2 in vitro. It is formally possible that the presence of replicating FXE in superinfected cultures induced the reactivation of latent HSV-2 but inhibited the subsequent multiplication of reactivated virus. However, this explanation for the failure of FXE or dl1403 to reactivate HSV-2 seems unlikely on the basis of previous experimentation in which HFL cells coinfectd with wt HSV-2 and dl1403 indicated that dl1403 did not interfere with HSV-2 replication (Russell et al., 1987).

4.2.3. Discussion.

The results presented in this section demonstrate that Vmw110 is the only HSV-1 gene product required for reactivation of HSV-2 in the in vitro latency system. Moreover, the domain affected by deletion of the cysteine-rich motif in Vmw110 is vital for reactivation. Transient transfection assays have indicated that Vmw110 is a potent transactivator (Everett, 1984; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a). Dissection of Vmw110 by inframe insertion/deletion mutagenesis has demonstrated that the region including FXE is important for the trans-acting function of this protein (Everett, 1988a) indicating that the events which precede reactivation are initiated by the activation of gene expression. The adenovirus recombinants do not encode the 5' portion of LAT, including the LAT

Figure 4.4. REACTIVATION OF LATENT HSV-2.

Experimental details are described in detail in the legend to Figure 4.3. Southern hybridization was carried out with a 655bp HSV-2 specific probe derived from a unique region of the glycoprotein G gene.

L, HSV-2

HSV-1 MOCK dl 1403 D14 MOCK FXE

M L L L L M L L L M L L L L M L L L

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

promoter, and D14 is simultaneously deleted for 41 amino acids in the C-terminus of the predicted LAT ORF1 (Figure 1.6.). Therefore, production of LAT is not necessary for reactivation since superinfection of latently infected monolayers with these viruses induces reactivation of HSV-2.

An extended analysis of the regions of Vmw110 important for reactivation in vitro has been reported using adenovirus mutants derived from the recombinant AdMLP-110 that express altered forms of Vmw110 (Zhu et al., 1990). In this study, mutations which affected the ability of Vmw110 to transactivate gene expression in transfection assays were defective for induction of reactivation. In particular, an adenovirus expressing Vmw110 with a deletion spanning the cysteine-rich motif failed to reactivate latent HSV-2, in agreement with the results presented in this thesis. Interestingly, a recombinant deleted for amino acids 628-697 in the carboxy terminus of Vmw110 failed to reactivate latent HSV-2 (Zhu et al., 1990). In contrast, mutant D14 defective in the same region retained the ability to stimulate the HSV-2 genome upon superinfection of latently infected monolayers (Figure 4.3. and 4.4.). It is plausible that the non-overlapping region defined by residues 628-680 may be responsible for the differing reactivation phenotypes of the two mutants. Alternatively, the combined effect of other HSV transactivators that are present during superinfection with D14 may compensate for other deletions in the carboxy end of Vmw110.

The mode of action of Vmw110 in the induction of reactivation remains unresolved. However, it is apparent that the ability of Vmw110 to reactivate latent HSV-2 distinguishes this polypeptide from the other HSV-1 transactivators, Vmw175, Vmw63 and Vmw65 which are present in a functional form during superinfection with dl1403 or FXE. Presumably, HSV-2 Vmw118 (the homologue of Vmw110), present in the endogenous latent genome, is inaccessible to the combined effect of Vmw175, Vmw63 and Vmw65 suggesting that the latent genome is in a novel quiescent state. It is postulated that due to the lack of target sequence specificity, Vmw110 may act directly on the latent genome by displacing DNA binding proteins from the genome, that is

organized in an inactive form resembling chromatin, thus rendering the genome accessible to cellular transcription factors. Alternatively, Vmw110 may function at a post transcriptional level by activating cellular genes whose products are responsible for reactivation.

Clearly, expression of Vmw110 is not required for reactivation of latent virus in vivo since Vmw110 deletion mutants capable of establishing a latent infection in mice can be reactivated by ganglionic explantation (Clements and Stow, 1989; Leib et al., 1989b). The logical explanation for these differing observations is that reactivation in vivo is triggered by cellular factors that mimic the action of Vmw110. Evidence supporting this hypothesis is provided by the demonstration that the efficiency of plaque formation of Vmw110 mutant viruses is increased significantly by growth on 2.5 BHK cells. 2.5 BHK cells were derived from repeated passage of BHK cells in medium normally used for HeLa cells, which causes a increase in growth rate and loss of contact inhibition (Everett, 1989). This suggests that 2.5 BHK cells can complement the mutants due to the presence of a cellular factor which can substitute for Vmw110 function and that is presumably absent or ineffective in BHK or HFL cells (Everett, 1989). Also, in contrast to the results presented in this thesis, mutants dl1403 and FXE were competent for reactivation upon induction of reactivation by NGF deprivation in an in vitro latency system based on the cultivation of rat sensory neurones (Wilcox et al., 1990; abstracts of the 15th International Herpesvirus Workshop, Washington DC, USA). These experiments imply that physiological alterations to neuronal cells in culture results in activation of a cellular factor able to compensate for defects in Vmw110 during reactivation.

4.3. Manipulation of Latently Infected Cultures.

The mechanism of reactivation was examined further by application of various treatments to latently infected cells.

4.3.1. Chemical Treatment of Latently Infected Monolayers.

A number of groups have used hypomethylating agents to increase the incidence and rate of reactivation from explanted neural tissue latently infected with HSV (Whitby et al., 1987; Bernstein and Kappes, 1988; Stephanopoulos et al., 1988). In this section, experiments are described in which latently infected cells exposed to the demethylating agents HMBA and DMSO were examined for reactivation of HSV-2.

The mode of action of DMSO and HMBA as DNA hypomethylating agents is unclear. The effects of HMBA include the induction of terminal differentiation of murine erythroleukaemia cells in a multistage process during which expression of a number of cellular transcripts is altered, particularly the down regulation of several proto-oncogenes (Ramsay et al., 1986) and accumulation of α and β globin, and rRNA transcripts (Sheffery et al., 1982; Cohen and Sheffery, 1985). Incorporation of 5mM HMBA into the growth media at explant of latently infected dorsal root ganglia or genital skin increased the frequency of recovery of HSV-2 (Bernstein and Kappes, 1988). DMSO is also known to have diverse effects on many cellular functions (Higgins, 1983; Rifkind et al., 1983). Application of undiluted DMSO on to the mouse skin is a potent inducer of reactivation of HSV in associated ganglia in vivo (Harbour et al., 1983). Explantation of latently infected mouse cervical ganglia and culture in the presence of 200mM DMSO resulted in the increased detection of virus reactivated from individual ganglia (Whitby et al., 1987). Other workers have employed DMSO to circumvent the failure to reactivate of certain Vmw110 deletion mutants (Leib et al., 1989b).

HFL cells were infected with HSV-2 (0.003 pfu/cell) and incubated at 42°C for 6 days followed by 4 days at 37°C. Growth media were replaced with EF5 or EF5 plus HMBA or DMSO at a variety of concentrations indicated in Table 4.2. Each parameter was assayed over 5 latently infected monolayers. After 3 days at 37°C monolayers were stained and examined for the presence of plaques. In contrast to the observations made using in vivo model systems, the data

Table 4.2. MANIPULATION OF LATENTLY INFECTED CULTURES.

TREATMENT ^a	CONDITIONS	PLAQUES PER PLATE ^b
HMBA:	0, 2.5, 5.0, 7.5, 10.0mM	0/5
DMSO:	0, 2.5, 5.0%	0/5
TRIGEMINAL GANGLION	+/- explanted trigeminal ganglion	0/5

a Cultures were infected with HSV-2 at 0.003 pfu/cell, incubated at 42°C for 6 days and then at 37°C for 4 days in EF5 prior to exposure to the treatments indicated. Each treatment was carried out as detailed in the text.

b The number of plaques per monolayer for each experimental condition. The results presented are from a single experiment although these have been repeated three and two times for HMBA and DMSO respectively with identical results.

presented in Table 4.2. indicate that incorporation of HMBA or DMSO over a range of concentrations failed to stimulate the reactivation of HSV-2 from its latent state in vitro.

4.3.2. Addition of Explanted Mouse Ganglia to Latently Infected Monolayers.

HFL cells were infected with HSV-2 at low moi (0.003 pfu/cell) and incubated in EF5 at 42°C for 6 days and then at 37°C for a further 4 days. EF5 was removed from monolayers and replaced with 2ml EF5 or 2ml EF5 containing a trigeminal ganglion dissected from a 4-6 week old BALB/c mouse. Monolayers were incubated at 37°C and observed for reactivation over a 7 day period. A 200µl aliquot of each sonicated cell harvest was assayed for the presence of infectious virus particles on 35mm plates of BHK cells in duplicate. Monolayers were overlaid with EHu2 and stained after 2 days at 37°C. The results presented in Table 4.2. show that reactivation of latent virus was not induced by addition of explanted neurological tissue in the growth media.

4.3.3. Discussion.

The results presented in this section describe attempts to experimentally reactivate HSV-2 by disruption of the latent state in vitro utilizing chemical and physical manipulations. However, each treatment proved to be unsuccessful in the stimulation of reactivation, although superinfection of latently infected cells treated with HMD at the NDT with 13 K₈m induced reactivation as expected. On the assumption that HMBA or DMSO treatment of latently infected cells demethylated the latent genome, then hypomethylation is apparently unable to induce sufficient HSV gene expression to produce lytic phase replication. This implies that latency in vitro is not a consequence of extensive methylation of the HSV genome and correlates with observations, using the same model system, that latent HSV-2 is not reactivated by inclusion of 5-azacytidine at concentrations between 0.75-6.0µM in the growth media (Russell, 1989). Furthermore, independent evidence has demonstrated that the latent HSV-1 genome is not heavily

methyalted in vivo (Deshmane and Fraser, 1987). In contrast, other in vivo findings suggest that 5-azacytidine, HMBA and DMSO induce reactivation of latent HSV from explants of neural tissue (Whitby et al., 1987; Bernstein and Kappes, 1988; Stephanopoulos et al., 1988). This apparent dichotomy may be explained if the documented pleiotropic effects of these hypomethylating agents involves the induction of a normally quiescent cellular function, absent or inactive in HFL cells, that presumably does not occur via demethylation.

It has been hypothesised that reactivation in vitro, by explantation of latently infected ganglia, may be induced by the release of a diffusable factor(s) produced upon the degeneration of the explanted tissue. The data presented in Table 4.2. indicates that addition of explanted neuronal tissue to the growth media of latently infected HFL cells did not reactivate HSV-2 over a 7 day period which is sufficient for reactivation in explanted ganglia latently infected with HSV (Stevens and Cook, 1971). This may be interpreted in either one of two ways: i) the theory is incorrect and soluble cellular factors are not responsible for reactivation in vitro, ii) or that latent HSV-2 during latency in tissue culture is refractory to the effect of prolonged exposure to these potential factors.

4.4. Characterization of Mutant In1814 in the In Vitro Latency System.

4.4.1. Establishment of a Latent Infection by In1814.

In the mouse eye model for latency in1814 establishes a latent infection in the trigeminal ganglia and reactivates from latency upon explant co-cultivation. Mutant in1814 was tested for the ability to establish latency in a tissue culture latency system.

4.4.1.1. Latent Infection with In1814 at Low MOI.

HFL cells were infected with in1814 or 1814R at 0.1 particle/cell (low moi) and following a 1hr absorption

period at 37°C the cultures were incubated at 42°C for 6 days and then at 37°C for 4 days. Superinfection of latently infected cultures with tsKsyn, followed by incubation at 38.5°C for 2 days in the presence of neutralizing serum, resulted in the appearance of non-syncytial plaques, demonstrating that reactivation of both 1814R and in1814 had occurred (Table 4.3.). Mock superinfected cultures did not yield infectious virus.

The data presented in Table 4.3. indicates that in1814 is able to establish a latent infection, despite the fact that the initial inoculum contained fewer pfu of in1814 (5) compared to 1814R (5500). Therefore, on the basis of particles, rather than pfu, in1814 established a latent infection as efficiently as 1814R.

4.4.1.2. Latency with In1814 at High MOI.

A problem inherent with the current in vitro latency system is that of low starting moi. The possibility of increasing the initial moi and hence the sensitivity of the latency system was investigated by exploiting the characteristic reduction in IE gene expression and growth of in1814 on HFL cells.

HFL cells were infected with 5, 2, or 1 particle of in1814/cell and incubated at 42°C for 6 days. During the incubation period at 37°C the growth medium was replaced with EHu2. After 2 days at 37°C the monolayers were stained and plaque numbers counted. Simultaneous with the establishment of latency the number of potential pfu within the initial inoculum was assessed by titration at 37°C on HFL cells.

Latency is established at 42°C when HFL cell cultures are infected at 5, 2, or 1 particle/cell of in1814. However, upon temperature downshift a small proportion of cells apparently harbour non-latent particles which, in the absence of Vmw65, replicate and form a plaque (Table 4.4.). Despite the presence of discrete plaques, the remaining HFL monolayer survived intact without obvious cytotoxicity. A 4-fold decrease in plaque number is observed for in1814 following incubation at 42°C for 6 days (Table 4.4.)

Table 4.3. ESTABLISHMENT OF LATENCY WITH In1814 AND 1814R.

SUPERINFECTION	INITIAL INFECTION ^a		
	MOI ^b	1814R	<u>in</u> 1814
Mock	-	0 ^c	0
<u>tsKsyn</u>	0.1	ND	200
	0.01	12	19
	0.001	2	ND

a Monolayers were infected with 0.1 particles/cell and incubated for 6 days at 42°C followed by 4 days at 37°C.

b Cultures were mock superinfected or superinfected at multiplicities of 0.01 and 0.001pfu/cell of tsKsyn, overlaid with EHu2 and stained after 2 days at 38.5°C.

c Non-syncytial plaque numbers/plate; expressed as a mean of 3 plates.

ND Not done.

Table 4.4. ESTABLISHMENT OF A HIGH MULTIPLICITY LATENT INFECTION.

INITIAL INFECTION ^a	AFTER TITRATION ^b	AFTER LATENCY ^c
5	27.0	7.4
2	10.5	2.6
1	6.0	1.4

a Latent infections were established in HFL cells at high moi using the standard in vitro latency protocol (Russell and Preston, 1986). Whilst at 37°C human serum was included in the growth medium.

b Titration of the inoculum was carried out at 37°C in parallel with establishment of latent infections; expressed as plaques/plate and a mean of 2 plates.

c Plaques/plate after the in vitro latency system; expressed as a mean of 5 plates.

...efficiently by superinfection ...
 ... The ...
 ...
 ...
 ...
 ...

indicating that 42°C treatment does not greatly reduce the titre of in1814.

4.4.1.3. Latency with In1814 at the Temperature Permissive for Virus Growth.

Mutant dl1403, which does not express a functional Vmw110, has a phenotype similar to in1814 in that growth is reduced in a cell dependent manner. Low multiplicity infections of HFL cells with dl1403 result in a non-productive state in which virus can be maintained at 37°C for several days, and virus can be reactivated by superinfection of monolayers with HCMV (Stow and Stow, 1989). In1814 was employed to investigate the possibility of establishing a latent infection at 37°C, the physiological temperature for virus growth.

HFL cells were infected with in1814 and latency was established at 42°C in the standard manner or directly at 37°C (without prior heat treatment) in the presence or absence of Ara-C. At various time points HFL cell cultures were mock superinfected or superinfected with 0.1 pfu/cell of tsKsyn or UV tsKsyn. Irradiation prevents detectable viral gene expression whilst retaining functional Vmw65. Therefore, superinfection with UV tsKsyn provides only Vmw65 whereas superinfection with unirradiated virus provides Vmw65 and the IE polypeptides, including Vmw110. Following incubation at 38.5°C for 2 days under EHu5 the monolayers were stained with giemsa and plaque numbers counted.

Throughout the time course experiment in1814 could be reactivated efficiently by superinfection of monolayers with tsKsyn (Table 4.5.). The in1814 genome could be recovered by superinfection with irradiated virus within the first 24hr of the experiment (Table 4.5.), which correlates with independent observations that functional Vmw65 supplied in trans complements in1814 (Ace et al., 1989). However, thereafter latent in1814 could not be reactivated by superinfection with UV tsKsyn (Table 4.5.), implying that as latency is established the IE promoter regions are modified and become unresponsive to transactivation by Vmw65. Despite these apparent alterations, the latent genome

Table 4.5. TIME COURSE FOR REACTIVATION OF LATENT In1814.

<u>Time of Superinfection</u> <u>(Days)</u>	<u>In1814 Pfu Reactivated</u> <u>after Superinfection^a</u>		
	Mock	UV <u>tsK</u>	<u>tsK</u>
<u>Latency at 42°C</u>			
0	<0.1	715	2500
1	<0.1	1.5	2015
2	<0.1	1.3	1960
3	0.5	0.4	2230
4	<0.1	<0.1	1350
<u>Latency at 37°C +Ara-C</u>			
0	2.4	35.5	3450
1	<0.1	<0.1	1610
2	<0.1	<0.1	1650
3	<0.1	<0.1	1420
4	<0.1	<0.1	1220
<u>Latency at 37°C -Ara-C</u>			
0	1	44.5	3720
1	<0.1	<0.1	1380
2	0.5	<0.1	1260
3	<0.1	<0.1	950
4	47	57	640

a HFL cells were initially infected with 10^6 , 10^5 or 10^4 particles per cell of in1814. At various time points post infection, HFL cell cultures containing latent in1814 were either mock superinfected or superinfected at 38.5°C with 0.1 pfu/cell UV tsKsyn or unirradiated virus. The amount of reactivated virus was determined in duplicate by plaque count and is expressed as pfu per 10^5 in1814 particles in the initial inoculum.

remains susceptible to transactivation by Vmw110.

After 4 days at 37°C, in the absence of Ara-C, latently infected monolayers showed signs of virus replication that probably represents spontaneous reactivation and replication of virus in a single plaque which is reported to occur on rare occasions after establishment of latency at 42°C (Russell and Preston, 1986).

In summary, these observations demonstrate that HFL cell cultures can be infected with a substantial number of in1814 particles at the temperature permissive for virus growth in analogy with observations of mutant dl1403 at 37°C (Stow and Stow, 1989). Moreover, the latent in1814 genome is differentially sensitive to HSV-1 transactivators. Incorporation of Ara-C in the growth medium did not affect the amount of virus that was reactivated upon superinfection (Table 4.5.).

4.4.1.4. Discussion.

Infection of HFL cells with in1814 at 42°C or 37°C results in the establishment of latency (Tables 4.3.-4.5.). Therefore, the disruption of Vmw65 function does not preclude establishment of a latent infection in vitro. The ability of in1814 to establish a latent infection at high multiplicity implies that the absence of Vmw65 predisposes HSV-1 to latency in cultured cells and which compares with the behaviour of in1814 in an animal model system (Steiner et al., 1990). In contrast, infection of HFL cells at high moi with dl1403, which has a similar growth phenotype to in1814 but produces wt levels of HSV-1 IE polypeptides except Vmw110, results in significant cell destruction. The differences observed between in1814 and dl1403, and the characteristic predisposition of in1814 to latent infections is presumably due to a very early block to viral gene expression in in1814 infections in which synthesis of IE gene products and cytotoxicity is drastically reduced.

After latency, a 10³ to 10⁴-fold decrease in titre is routinely seen by 42°C treatment of HFL cells infected by HSV-2 (Russell, 1989). In comparison, only a 4-fold reduction in plaque number was observed for in1814 at 42°C

(Table 4.4.) implying that the combined effect of mild heat shock of HFL cells and the mutation are not additive. Therefore, 42°C treatment of HFL cells and disruption of Vmw65 function may exert their effects through the same pathway involving IE gene expression. Incubation of HFL cells at the supraoptimal temperature of 42°C induces the cellular heat shock response (Notarianni, 1986; Figure 4.9.) which affects the transcription of several cellular genes. Therefore, the effect of 42°C treatment may be manifest at the level of OTF-1 transcription, or any of the other cellular transcription factors which interact with Vmw65 in IEC formation. Alternatively, mild heat shock may influence viral gene expression directly at the level of Vmw65, perhaps by modifying its binding affinity for cellular components.

It is not clear from these data if the small proportion of effective particles which form a plaque after the in vitro latency system (Table 4.4.) are produced by replication in a subpopulation of cells capable of supporting virus growth in the absence of Vmw65 function or represent a Poisson distribution of the actual number of particles received by an individual cell such that a threshold level is exceeded and a gene dosage effect permits the formation of a plaque.

The particle:pfu ratio of in1814 stocks varies with the cell state. Particle:pfu ratios in the order of approximately 2×10^5 have been observed (Ace et al., 1989). However, in1814 routinely has a particle:pfu ratio in the range of 10^4 - 5×10^4 on HFL cells. Therefore, by extrapolation, application of 5 particles/cell of in1814 to a confluent monolayer on a 35mm petri dish, which averages 10^6 cells/plate, would routinely result in the formation of 100-500 plaques. Incorporation of Ara-C into the growth medium suppresses the negligible background of non-latent virus particles (0.05-0.5%) present amongst the majority of latent particles, thus producing a stable high multiplicity latency system at 42°C or 37°C. Indeed, results presented in Table 4.5. and, Section 4.4.2. and 4.4.4. indicate that at 37°C with or without Ara-C the virus-cell interaction is indistinguishable from latency at 42°C.

Table 4.5. indicates that during latency IE promoters undergo rapid alterations such that they are refractory to stimulation by Vmw65. This contrasts with HSV productive infections in which the the response of IE control regions to Vmw65 is central to progression to full lytic phase gene expression. Thus, during latent infections the IE promoters are apparently only responsive to Vmw110, which fits with the observation that reactivation, in the in vitro latency system employed here, relies on Vmw110. The phenomena may be related and occur for the identical reason. For example, if the latent genome is packaged into chromatin then Vmw110 may non-specifically displace bound nucleosomes, rendering the genome available to the cellular transcription machinery. Alternatively, this may reflect the presence of a specific inhibitor of IE transcription in HFL cells analogous to that claimed to exist in a mouse neuroblastoma cell line (Kemp et al., 1990).

4.4.2. The State of the Latent In1814 Genome.

4.4.2.1. Introduction.

Mouse and human neurological tissues latently infected with HSV-1 contain DNA that lack\$terminal restriction endonuclease fragments (Rock and Fraser, 1983; Efsthathiou et al., 1986). Thus, latent HSV-1 genomes are in a physical state that differs from the characteristic unit length molecules found in virion DNA. The current working model is that the latent HSV-1 genome is probably in the form of a circular episome which is associated with nucleosomes in a chromatin-like arrangement (Rock and Fraser, 1983; Mellerick and Fraser, 1987; Deshmane et al., 1989). In contrast, HEL cells latently infected in vitro with HSV-1 have been shown to contain only unit length linear viral DNA (Wigdahl et al., 1984a).

Analysis of the physical nature of the latent in1814 genome in the in vitro latency system was enhanced by the infection of HFL cells at high multiplicity (5 particles/cell). Since the predominant form of HSV-1 genomes during latency in vivo is non-linear, it was

important to investigate the molecular organization of in1814 DNA in the in vitro latency to facilitate comparison with other independent in vivo and in vitro observations.

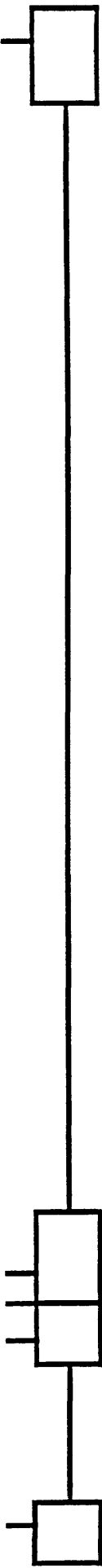
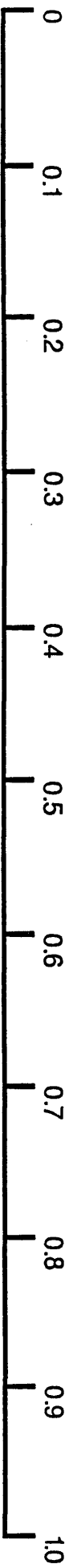
4.4.2.2. Latent In1814 DNA is in a Non-Linear Configuration.

The physical organization of the HSV genome was analyzed in HFL cell cultures infected with in1814 at 5 particles/cell and incubated at 42°C or 37°C in the presence of 50µg/ml Ara-C or 5µg/ml aphidicolin. Nuclear DNA was extracted from cultures at 5hr, 2 days or 4 days post infection. Samples were digested with BamHI and subjected to density gradient centrifugation to remove the majority of cellular DNA sequences, thereby enriching for HSV fragments. DNA restriction fragments with densities ranging from 1.709 to 1.746 were separated by gel electrophoresis and analyzed by Southern hybridization utilizing a HSV-1 strain 17 BamHI k probe labelled to high specific activity (Figure 4.5.). In reconstruction experiments with genomic HSV-1 DNA, the presence of the joint spanning fragment (BamHI k) and the terminal fragments (BamHI q and s) were detected. In nuclei extracted 5hr after infection, hybridization to the BamHI fragments k, q and s was apparent, although BamHI q and s were present at reduced levels compared to BamHI k (Figures 4.6.-4.8.). In contrast, samples extracted at 2 and 4 days post infection were notable for the absence of hybridization to BamHI q and s, but the presence of hybridization to the joint sequence BamHI k regardless of whether latency was established at 42°C or at 37°C with DNA replication inhibitors (Figures 4.6.-4.8.). It is unlikely that the lack of BamHI terminal sequences is due to the DNA purification protocol as it has been demonstrated that the terminal fragments are not lost during the processing of DNA samples which contain a mixture of BamHI digested mock infected HFL cell DNA and viral genomic DNA (Russell, 1989; S. Jamieson, unpublished observation).

HFL cells infected with in1814 and incubated at 42°C or 37°C contain the HSV-1 joint fragment but do not contain detectable amounts of the BamHI terminal fragments. Also,

Figure 4.5. THE GENOMIC LOCATIONS OF THE HSV-1 RESTRICTION
FRAGMENTS BamHI k, q AND s.

The HSV-1 BamHI fragments illustrated in the diagram are the joint fragment BamHI k ($k=q+s$), and the terminal BamHI fragments q and s. The scale in mu is indicated.



s

s q

q

$$k=s+q$$

Figure 4.6. DETECTION OF In1814 DNA AT 42°C.

Lanes 1-3 contain 5, 1.5, and 0.5ng of BamHI digested HSV-1 genomes respectively. Nuclear DNA of densities 1.709 to 1.746 were extracted from mock infected HFL cells after 4 days at 42°C (lane 4) and latently infected cultures which had been incubated at 42°C for 5hr (lane 5), 2 days (lane 6) or 4 days (lane 7). Electrophoresed DNA restriction products were analyzed by Southern hybridization with a ³²P-labelled BamHI k probe. Positions of BamHI k (joint fragment) and BamHI q and s (terminal fragments) are indicated.

5 1.5 0.5 M 5h 2d 4d
1 2 3 4 5 6 7

K

Q
S

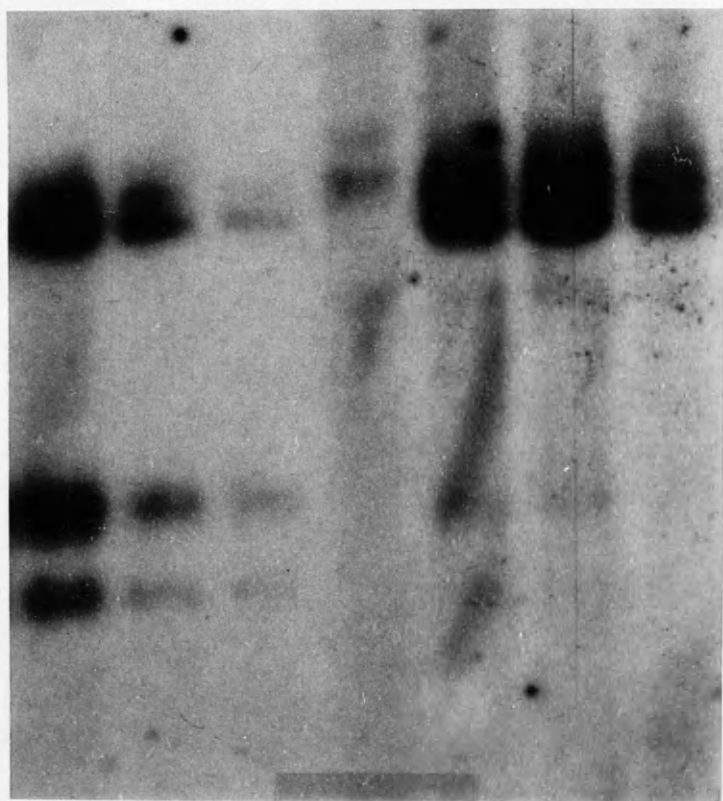


Figure 4.7. LATENT In1814 DNA AT 37°C WITH ARA-C.

Experimental details are described in the legend to Figure 4.6. except that latent DNA was extracted from cells incubated at 37°C with the inclusion of 50µg/ml Ara-C in the growth medium.

5 1.5 0.5 M 5h 2d 4d
1 2 3 4 5 6 7

K

Q

S

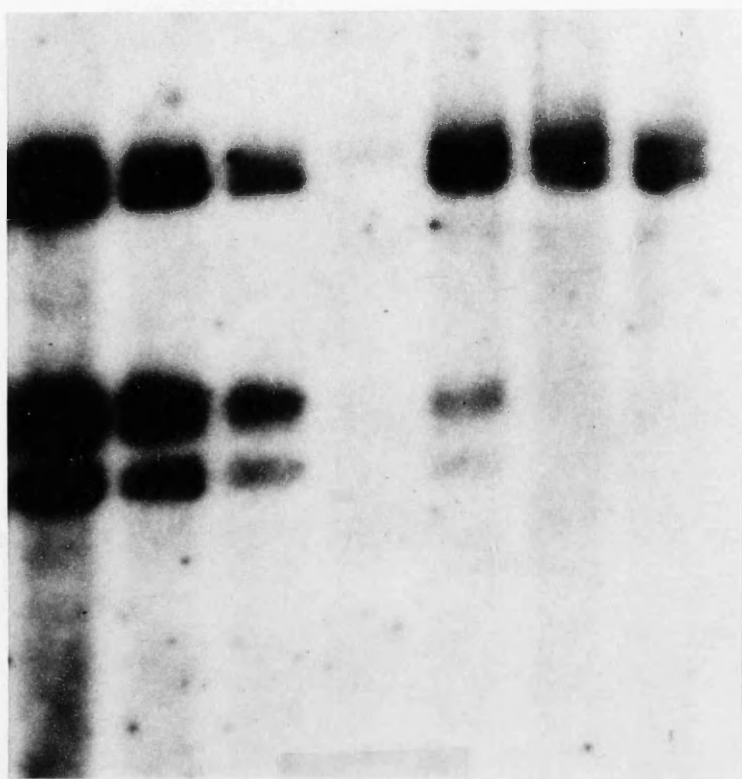


Figure 4.8. LATENT In1814 DNA AT 37°C WITH APHIDICOLIN.

Experimental details are as for Figure 4.6. with the exception that DNA was extracted from HFL cells that had been incubated at 37°C in the presence of 5µg/ml aphidicolin.

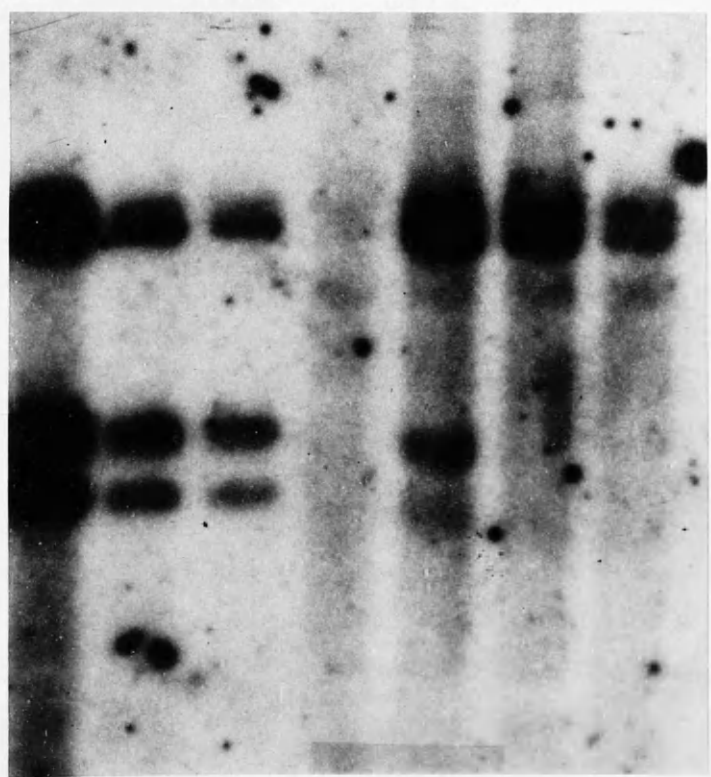
The absence of signal at 1750 is not a product of a specific inhibitor as additional samples were subjected with Ac2O and pyridine. Therefore, during polymerization, the polymerization is not inhibited by the polymerization.

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1.1.10⁷
of 10⁷
1.1.10⁷
results
1.1.10⁷

5	1.5	0.5	M	5h	2d	4d
1	2	3	4	5	6	7

K

Q
S



the absence of termini at 37°C is not a product of a specific inhibitor as identical results were obtained with Ara-C and aphidicolin. Therefore, during latency in vitro in1814 DNA exists in an "endless" conformation. The DNA may persist as a unit length molecule integrated at a unique site into the cellular genome via the terminal repeats which would generate novel sized terminal restriction fragments. Since this type of fragment was not detected, the results presented in Figures 4.6.-4.8. indicate that the predominant form of the in1814 genome during latency in tissue culture must be either a circular or concatemeric molecule, or integrated into the cellular genome via regions of the viral genome excluding the termini.

The latent genome copy number was calculated by comparison of the hybridization signals obtained for reconstruction experiments (Figure 4.6-4.8, lanes 1-3) with latently infected DNA samples. The control samples contained 5, 1.5 and 0.5ng BamHI digested viral genomes which is assumed to represent 2×10^7 , 6×10^6 and 2×10^6 HSV genomes per 10^6 cells respectively. Densitometric analysis (Section 3.7.5.) showed that in a DNA extract derived from 1.5×10^7 HFL cells initially infected with 5 particles/cell in1814, the HSV-1 joint fragments were detected in the range of approximately 0.1-0.5 copies/cell at 37°C and 0.2-1.0 copies/cell at 42°C in a monolayer of 10^6 HFL cells.

4.4.4.3. Discussion.

Infection of HFL cell monolayers with 5 particles/cell of in1814 and incubation at the supraoptimal temperature of 42°C or directly at the physiological temperature 37°C results in the conversion of the HSV genome to an "endless" molecule. The proportion of termini present in the latent DNA samples is decreased by 5hr post infection and apparently undetectable 2 days later. Therefore, latent genomes are converted rapidly to a non-linear state.

Infectious centre assays estimate that between 13% and 21% of cells receive a potentially active genome following infection of 10^6 HFL cells with 5×10^6 in1814 particles (C.M. Preston, unpublished observations). Therefore, at most

approximately 2.0×10^5 pfu could be recovered from the initial inoculum. Densitometric analysis of hybridization signals obtained with reconstruction experiments relative to latently infected samples suggests that 0.1-1.0 joint fragments per cell are present in a monolayer of 10^6 cells. Thus, each latently infected cell retains a maximum of 1-5 in1814 genomes. Furthermore, the amount of joint DNA detected remained constant or decreased implying that, over the 4 day period examined, replication of viral DNA did not occur. Thus, it is unlikely that the presence of "endless" DNA is due to concatemeric structures formed by a rolling circle mechanism. In summary, the results presented in this section demonstrate that non-linear HSV-1 DNA is maintained at low abundance in an in vitro latency system. This correlates well with an independent study in which infection of HFL cells with wt HSV-2 at the supraoptimal temperature also resulted in the conversion of the genome to an "endless" molecule present at low copy number (Preston and Russell, 1991). Moreover, in the same study, joint fragments were detected at bimolar concentrations relative to a unique region of the genome demonstrating that the lack of terminal fragments was due to the covalent fusion of termini to form an additional joint fragment. Thus, HSV-2 DNA during latency in vitro is predominately circular, concatenated or integrated via internal regions (Preston and Russell, 1991). By comparison with these data and other in vivo observations which suggest that latent HSV DNA is in a circular form (Mellerick and Fraser, 1987) it is postulated that in1814 is present as a circular episome during latency at 42°C or 37°C.

Despite increasing the initial MOI of the in vitro latency system, it is not clear why infection of HFL cells with a relatively high multiplicity of in1814 compared to wt HSV-2 yields an equivalent number of latent genomes per HFL cell (Preston and Russell, 1991). Possibly HFL cells can tolerate a finite level of latent HSV genomes. However, this is not compatible with in vivo observations which suggest that the HSV genome may be present in the order of several hundred copies per latently infected neuronal cell.

A related in vitro latency system has been described in which viral replication is repressed at 37°C by treatment of HEL cell monolayers with the antiviral agents BVDU and IFN- α (Wigdahl et al., 1982b). However, in this system latent HSV-1 DNA is principally unit length and linear (Wigdahl et al., 1984b). Thus, despite obvious similarities the two systems must be fundamentally different at the molecular level. This difference may be ascribed to the existence of two subpopulations of latently infected cells within a HEL cell monolayer (Shiraki and Rapp, 1986). One subpopulation showed signs of spontaneous reactivation after temperature downshift to 37°C. In the other subpopulation, reactivation was dependent on superinfection of latently infected cultures with HCMV. The proportion of cells that reactivate upon temperature reduction decreased rapidly with time whilst the number that produced infectious virus after superinfection remained constant (Shiraki and Rapp, 1986). Thus, it is possible that the more stable form of virus found at 37°C may represent a minor undetectable DNA species that is non-linear.

In conclusion, in nuclear extracts from latently infected HFL cells at 42°C or 37°C in1814 DNA is in a form other than unit length molecules. This is the first evidence that HSV-1 DNA is non-linear in vitro and correlates with available information pertaining to the physical structure of the latent HSV-1 genome in mouse model systems (Rock and Fraser, 1983; Efsthathiou et al., 1986).

4.4.3. Viral Gene Expression During Latency.

Several approaches were adopted to examine the status of latent in1814 genome expression in the in vitro latency system. These included the use of an enzyme assay to detect the synthesis of products from an early HSV gene, the analysis of polypeptide profiles and PCR amplification of latently infected cellular RNAs.

4.4.3.1. Analysis of Latently Infected Monolayers for Viral TK Activity.

TK is a prototype HSV early gene in terms of its promoter and regulatory regions. The TK assay employed permits the sensitive detection of viral TK enzymatic activity.

TK activity was assessed in HFL cells that had been mock infected or infected with 5 particles/cell in1814 or 1814R. Monolayers were incubated at 42°C or 37°C and cytoplasmic extracts were prepared at various time points between 3hr and 96hr post infection. The results of TK assays performed on cell extracts (Table 4.6.) indicate that viral TK activity in cells infected with in1814 was not significantly different from values obtained with mock infected extracts at 42°C and 37°C. A similar pattern was observed in 1814R infected cultures at 42°C. However, TK enzymatic activity was increased after infection of HFL cells with 5 particles/cell 1814R and incubation at 37°C for 9-48hr.

Therefore, the expression of TK and presumably other HSV early genes is restricted in latently infected cultures at 42°C implying that a functional TK polypeptide is not essential for maintenance of the latent state in vitro. Transcription of the TK gene during latency in vitro is investigated in Section 4.4.3.

4.4.3.2. Latent Infection Polypeptide Profiles of In1814 and 1814R.

To determine which, if any, virus specific polypeptides are synthesised during latency, HFL cell cultures were mock infected or infected with in1814 or 1814R at 5 particles/cell. Cultures were pulse-labelled for 1hr after incubation at 42°C or 37°C for varying lengths of time. Labelled, infected cell polypeptides were analyzed by SDS polyacrylamide gel electrophoresis. The results shown in Figure 4.9. indicate that the polypeptide profile of the mutant virus is similarly restricted at 42°C and 37°C. 1814R protein synthesis is likewise restricted at 42°C, but

Table 4.6. TIME COURSE OF TK GENE EXPRESSION WITH In1814 AND 1814R DURING LATENT INFECTIONS IN VITRO.

<u>Time at 42°C</u> (hr)	<u>TK Activity</u> (cpm/μg protein)		
	Mock	<u>in</u> 1814	1814R
3	7.0	2.4	3.0
6	5.9	2.7	3.3
9	4.5	3.7	3.9
18	5.1	2.7	3.4
24	3.7	4.1	6.8
36	3.9	3.6	4.0
48	4.7	4.5	1.8
72	5.5	8.9	6.4
96	5.5	6.5	3.7

<u>Time at 37°C</u>			
9	5.4	4.8	19.6
24	4.9	4.4	33.9
36	4.0	2.3	65.0

HFL cells were mock infected or infected with in1814 or 1814R at 5 particles/cell and incubated at 42°C or 37°C. Cultures were harvested at the time point indicated. TK activity was determined by assay of the cytoplasmic fraction from each sample.

Figure 4.9. VIRAL POLYPEPTIDES DURING LATENCY AT 42°C.

HFL cells were mock infected (lanes 1 to 5), infected with 5 particles/cell in1814 or 1814R (lanes 6 to 10; lanes 11 to 15 respectively). Cultures were pulse labelled after incubation at 42°C (lanes 1 to 4, lanes 6-9 and lanes 11 to 14) or 37°C (lanes 5, 10 and 15) for 3hr (lanes 1, 6 and 11), 6hr (lanes 2, 7 and 12), 9hr (lanes 3, 5, 8, 10, 13 and 15) or 16hr (lanes 4, 9 and 14). HSV-1 induced polypeptides are indicated •. A cellular heat shock protein is labelled □.

as expected during incubation at 37°C some virus specific polypeptides are visible.

4.4.2.3. PCR Analysis of Latent Gene Expression.

During HSV-1 latent infections gene expression is restricted to a family of transcripts that have been detected in mice, rabbits and humans (Spivack and Fraser, 1987; Stevens et al., 1987; Rock et al., 1987a; Croen et al., 1987; Deatly et al., 1987,1988; Wagner et al., 1988b; Krause et al., 1988; Steiner et al.,1988; Weschler et al., 1988b; Gordon et al., 1990a).

PCR is an in vitro method for the primer-directed enzymatic amplification of specific nucleotide sequences that is capable of producing large amounts of a defined fragment from small quantities of a target sequence (Saiki et al., 1988). PCR amplification of cellular RNAs has been adapted to study the status of gene expression from the latent HSV-1 genome in murine cervical ganglia (Lynas et al., 1989a,b). The PCR technique facilitated the detection of LAT gene expression in <1µg of RNA extracted from the combined ganglia of several latently infected animals which corresponds approximately to the estimated RNA content of an individual ganglion (Lynas et al., 1989b).

The production of LAT and TK mRNA was examined in HFL cells latently infected with in1814 and incubated at 42°C or 37°C +/- Ara-C for 4 days. Total RNA extracted from infected HFL cells was treated with RNase-free DNase to remove contaminating DNA, and was converted to cDNA using the appropriate antisense oligonucleotide primer (LAT 2, TK 2). The products of the cDNA synthesis reaction were then subjected to 30 cycles of PCR amplification with the addition of the sense oligonucleotide (LAT 1, TK 1) 5' end labelled with ³²P. Incorporation of an end-labelled primer during the PCR amplification permits the specific quantitative detection of low abundance RNAs (Arrigo et al., 1989).

Parallel analysis of samples with 5' oligonucleotide primers in the cDNA synthesis and 3' primers in the PCR amplification permits the discrimination of a DNA signal from an RNA signal since DNA will generate a product with either

combination of primers whereas RNA is detected specifically with the mRNA complementary (3' or antisense) primer during the cDNA synthesis reaction.

The LAT oligonucleotide primers utilized originate from the 3' end of LAT in a region that is unique to LAT which is not complementary to Vmw110 and does not involve the putative 3' intron detected by Wechsler et al. (1988a) (Figure 4.1.). Amplification of a 132bp fragment is indicative of transcription from the region encoding LAT.

The TK specific primers are not identical but overlap the primers chosen by Lynas et al. (1989a,b) and the 3' oligonucleotide primer is located close to the TK polyadenylation signal. PCR analysis of RNA samples containing TK mRNA is expected to yield an amplified product of 104bp.

The LAT specific fragment was amplified from virion DNA and a BHK cell extract made at late times post infection (Figure 4.10). However, the LAT PCR product is not detected in HFL cells infected with in1814 at high multiplicity at 42°C and 37°C or as expected in a mock infected extract made from cells incubated at 42°C for 4 days (Figure 4.10.). Likewise, the TK PCR fragment could be detected in DNA extracted from virus particles or in BHK cells at late times post infection but in HFL cells TK gene expression was not detectable during latent infections (Figure 4.11.). PCR examination of RNA extracted from HFL cells infected with 1814R for 20hr at 37°C revealed the presence of PCR products using the HSV LAT and TK specific primers in both combinations implying that the positive result may be due to contaminating DNA (Figures 4.10. and 4.11.). This presumably also accounts for the presence of a positive signal in the mock infected extract subjected to PCR amplification in the presence of TK 2 (Figure 4.11. lane 5). These data suggest that during maintenance of latency in vitro expression of HSV genes, including LAT, does not occur.

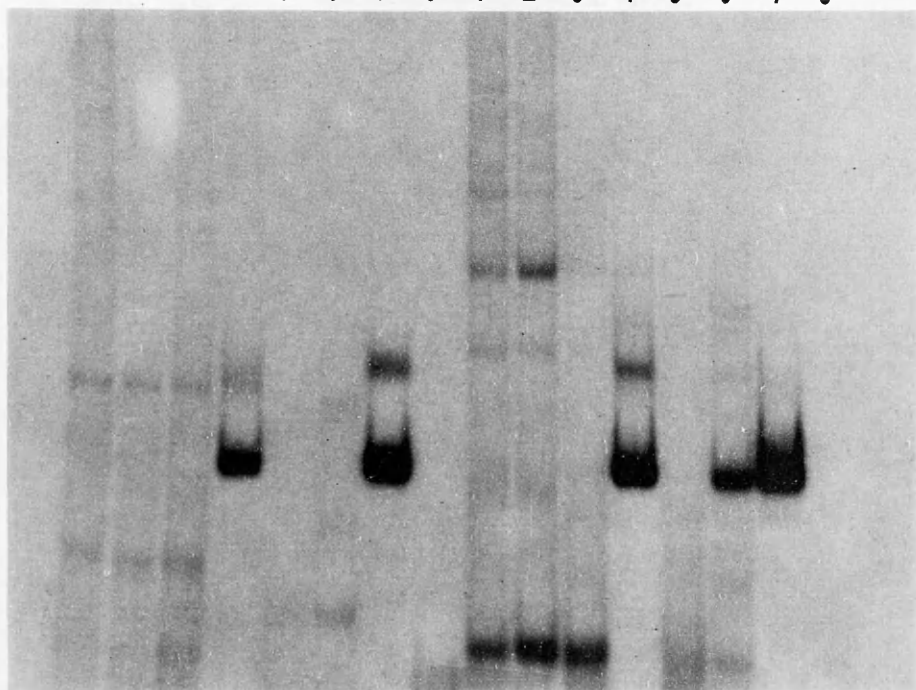
Figure 4.10. PCR ANALYSIS OF LATENTLY INFECTED HFL CELL RNAs
USING LAT SPECIFIC PRIMERS.

HFL cells were either mock infected (lane 1), infected with 5 particles/cell in1814 and incubated at 42°C for 4 days (lane 2), 37°C for 4 days with 50µg/ml Ara-C (lane 3) or 5 particles/cell 1814R and incubated for 20hr at 37°C (lane 4). Total cell RNA was isolated using guanidinium thiocyanate, and treated with RNase-free DNase. Lanes 5 and 6 contain a mock infected cell extract and a late extract respectively. Lane 7 is the amplified product from 200ng of HSV-1 DNA. Lane 8 contains no PCR template. RNA samples were converted to cDNA using the 5' (sense) or 3' (antisense) oligonucleotide primer (Figure 4.10 A and 4.10 B respectively) and were subjected to PCR after addition of the complementary end-labelled primer. Products were separated on a 6% polyacrylamide gel and visualized by autoradiography. The position of the 132bp LAT specific fragment (●) is indicated relative to non-radioactive size markers.

A

B

1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8



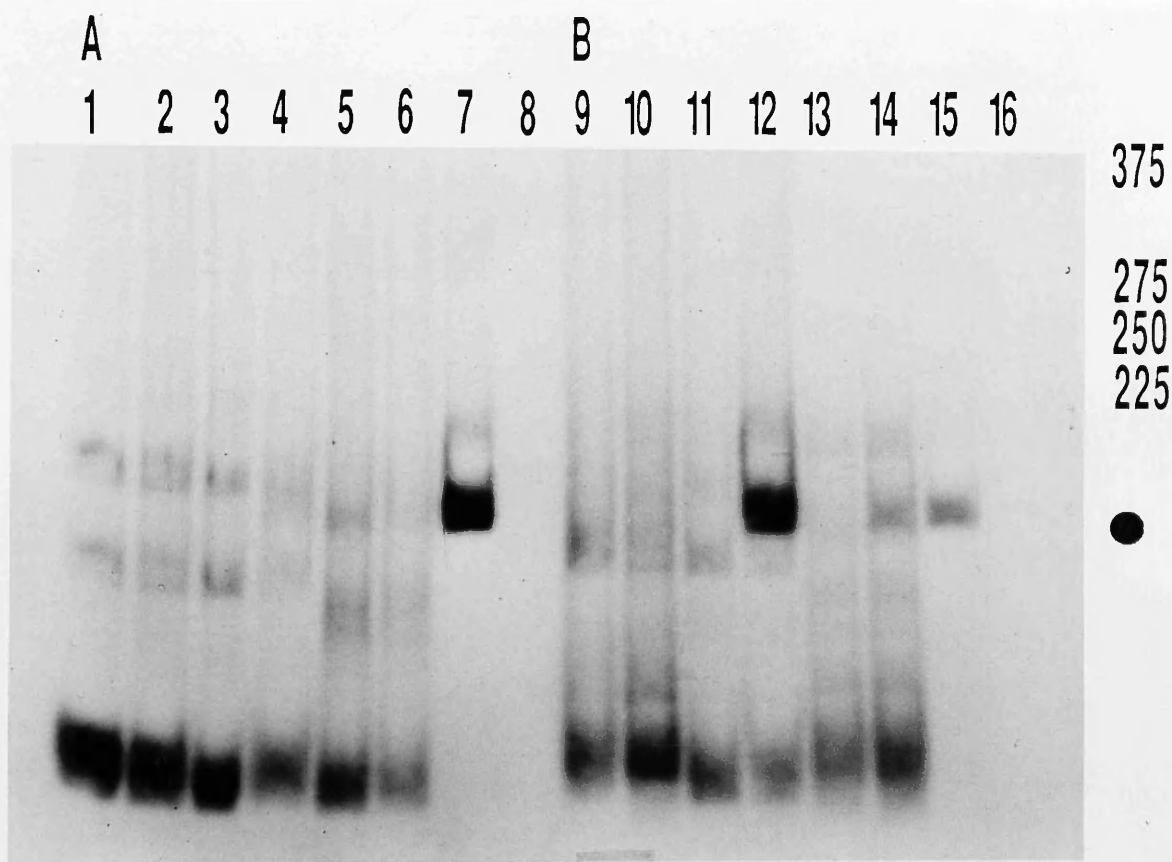
375
275
250
225

Figure 4.11. PCR ANALYSIS OF LATENTLY INFECTED HFL CELL RNAs
USING TK SPECIFIC PRIMERS.

HFL cells were either mock infected (lane 1 and 9), infected with 5 particles/cell in1814 and incubated at 42°C for 4 days (lane 2 and 10), 37°C for 4 days with 50µg/ml Ara-C (lane 3 and 11) or 5 particles/cell 1814R and incubated for 20hr at 37°C (lane 4 and 12). Total cell RNA was isolated using guanidinium thiocynate, and treated with RNase-free DNase. Lanes 5, 13 and 6 and 14 contain a mock infected cell extract and an extract harvested at late times post infection respectively. Lanes 7 and 15 are the amplified product from 200ng of HSV-1 DNA. Lane 8 and 16 contain no template. RNA samples were converted to cDNA using the 5' (sense) or 3' (antisense) TK oligonucleotide primer (Figure 4.11 A and 4.11 B respectively) and were subjected to PCR after addition of the complementary end-labelled primer. Products were separated on a 6% polyacrylamide gel and visualized by autoradiography. The position of the 104bp TK specific fragment (●) is indicated relative to size markers.

4.2.4. Discussion.

A characteristic of latent infections is that the suppression of the host's immune response is essential for the virus to persist and multiply. In the case of the herpesviruses, the virus genome is integrated into the host genome and the virus is able to evade the host's immune response by a variety of mechanisms. The virus is able to suppress the host's immune response by a variety of mechanisms, including the inhibition of the host's immune response by the virus genome. The virus is able to suppress the host's immune response by a variety of mechanisms, including the inhibition of the host's immune response by the virus genome.



The results of the gel electrophoresis experiment are shown in Figure 4.2.4. The results show that the virus is able to suppress the host's immune response by a variety of mechanisms, including the inhibition of the host's immune response by the virus genome. The virus is able to suppress the host's immune response by a variety of mechanisms, including the inhibition of the host's immune response by the virus genome. The virus is able to suppress the host's immune response by a variety of mechanisms, including the inhibition of the host's immune response by the virus genome.

4.4.2.4. Discussion.

A characteristic of latent infections in vivo is the suppression of transcription from the majority of viral genes. Only a region encompassing the terminal repeats is actively expressed. Although the subject of intense research, the biological function(s) of the LAT gene products have not been determined. The analysis of engineered and spontaneously arising LAT mutant viruses in animal model systems indicate that LAT gene expression is not required for establishment or maintenance of latency (Javier et al., 1988; Steiner et al., 1988; Leib et al., 1989a; Sedarati et al., 1989). Therefore, the importance of LAT is speculated to be in events that lead to reactivation. Indeed, a recent study has suggested that LAT may be required in reactivation in vivo induced by iontophoresis of epinephrine (Hill et al., 1990).

Examination of the status of latent gene expression utilizing the in vitro latency system suggests that, like the in vivo situation, viral gene expression is restricted. In contrast to observed in vivo phenomena LAT gene products are undetectable during in1814 latent infections at 42°C or at 37°C. This is presumably not a consequence of an inability of in1814 to synthesize LAT since latent LAT gene expression has been detected by in situ hybridization analysis of mouse trigeminal ganglia (Vlayi-Nagi et al., 1991). An explanation for the absence of LAT in vitro may reside in the apparent increase in activity of the LAT gene promoter in cells of neuronal origin (Batchelor and O'Hare, 1990; Zwaagstra et al., 1990). The specificity of the LAT promoter in neuronal-derived cells is thought to reside in an element which lies between nucleotides -797 to -1267 relative to the LAT RNA start site. Furthermore, gel retardation analysis has detected a nuclear protein present in neuronal and non-neuronal cells that binds upstream of the TATA box and appears at least partly responsible for promoter activity in in vitro assays (Zwaagstra et al., 1990, abstracts of the 15th International Herpesvirus Workshop, Washington DC, USA). The absence of such cellular factors, which may include proteins capable of interaction

with the neuronal specific control region, in HFL cells could prevent the expression of LAT. Alternatively, the lack of LAT during maintenance of the latent infection in vitro may be a consequence of technical failure. The availability of a positive control sample isolated from latently infected ganglia or amplification of an internal cellular control, such as actin sequences, would have eliminated this latter explanation.

In the context of a previous report, it is perhaps not surprising that TK enzymatic activity was not detected during in1814 latency in vitro (Russell, 1989). In this study it was postulated that the transcription, translation or processing of the enzyme may be inhibited in HFL cells latently infected with HSV-2, or alternatively that functionally important domains of TK may be altered in such a way as to prevent enzymatic activity in this situation. PCR analysis of in1814 latency in HFL cells (Figure 4.11.) implies that the block to TK, and probably other HSV-1 genes, gene expression is at the level of transcription since TK specific mRNA was not detected. Likewise, using a similar approach, Lynas et al., (1989b) found no evidence of TK transcripts during latent infections in vivo.

In conclusion, during in1814 latent infections there appears to be very limited, if any, viral gene expression. Therefore, in common with other in vitro and in vivo observations the latent in1814 genome is rendered transcriptionally silent during latent infections. The obvious difference between the quiescent state in vivo and that reported here is the apparent lack of LAT. Non-production of LAT is not a universal feature of in vitro latency models since LAT is readily detected in an other in vitro latency system based on the cultivation of primary rat neurones (Doerig et al., 1990, abstracts of the 15th International Herpesvirus Workshop, Washington DC, USA) which supports the hypothesis that the failure to detect LAT by PCR is most likely due to the neuronal specificity of the LAT promoter.

In vivo, LAT is not required for establishment or maintenance of latency, and its precise role in reactivation remains to be ascertained. The absence of LAT does not

therefore invalidate latency studies in vitro, but future work may reveal subtle differences in the molecular events that contribute to the reactivation process.

4.4.4. Reactivation of Latent Virus.

4.4.4.1. Introduction.

It is apparent that the latent HSV genome is responsive to a variety of stimuli which induce reactivation in vivo (Section 1.3.4.). However, the molecular mechanisms involved in this process have not yet been elucidated.

Reactivation of latent HSV-2 from an in vitro latency system that depends on infection of cells in the presence of antiviral agents for establishment, followed by incubation at increased temperature (40.5°C) for maintenance, can be experimentally induced to reactivate by superinfection of monolayers with HSV ts mutants or HCMV DNA-negative ts mutants which synthesise only IE and early gene products at the NPT (Colberg-Poley et al., 1979b,1981; Wigdahl et al., 1982a,b).

During latency in the in vitro latency system employed in this thesis, the virus-cell interaction is not perturbed by chemical or physical manipulations of latently infected cultures (Russell, 1989; Section 4.3.). However, reactivation of HSV from its latent state can be induced by intertypic superinfection with wt HSV-1, HCMV or HSV mutant viruses that produce the IE polypeptide Vmw110 (Russell et al., 1987; Section 4.2.). Furthermore, disruption of a 45 amino acid domain in Vmw110 is sufficient to preclude reactivation induced by HSV superinfection (Section 4.3.).

4.4.4.2. Reactivation of Latent HSV-2 by In1814.

To confirm that the virion transinducing factor Vmw65 was not required for reactivation, mutant in1814 was tested for its capacity to reactivate latent HSV-2 upon superinfection. Cultures were infected with wt HSV-2 (0.003 pfu/cell) and incubated for 6 days at 42°C followed by 4 days at 37°C. Latently infected monolayers were

superinfected over a range of multiplicities (0.001-0.1 particles/cell) with wt HSV-1, 1814R or in1814 and incubated at 37°C for a further 2 days. Cytoplasmic DNA, extracted from BHK cells infected with 100 μ l of the HFL cell harvest, was digested with HindIII and analyzed by hybridization of a Southern blot with a ³²P-labelled HindIII L fragment (Figure 4.2.).

Figure 4.12. indicates that each superinfecting virus replicated efficiently and induced the reactivation of latent HSV-2 as demonstrated by the presence of the HSV-2 specific HindIII L band against a background of cross-hybridizing HSV-1 bands HindIII b, e, g and n. However, reactivation was consistently reduced in latently infected monolayers superinfected with in1814. In BHK cells infected with in1814 the amount of Vmw110 mRNA is reduced 4 to 5-fold and importantly, a 10-fold decrease in the level of Vmw110 is observed in in1814 infected HFL cells (Ace et al., 1989). The importance of Vmw110 in reactivation is discussed in Section 4.2. and it is probable that the reported reduction in Vmw110 transcription and translation is sufficient to decrease the reactivation efficiency of in1814.

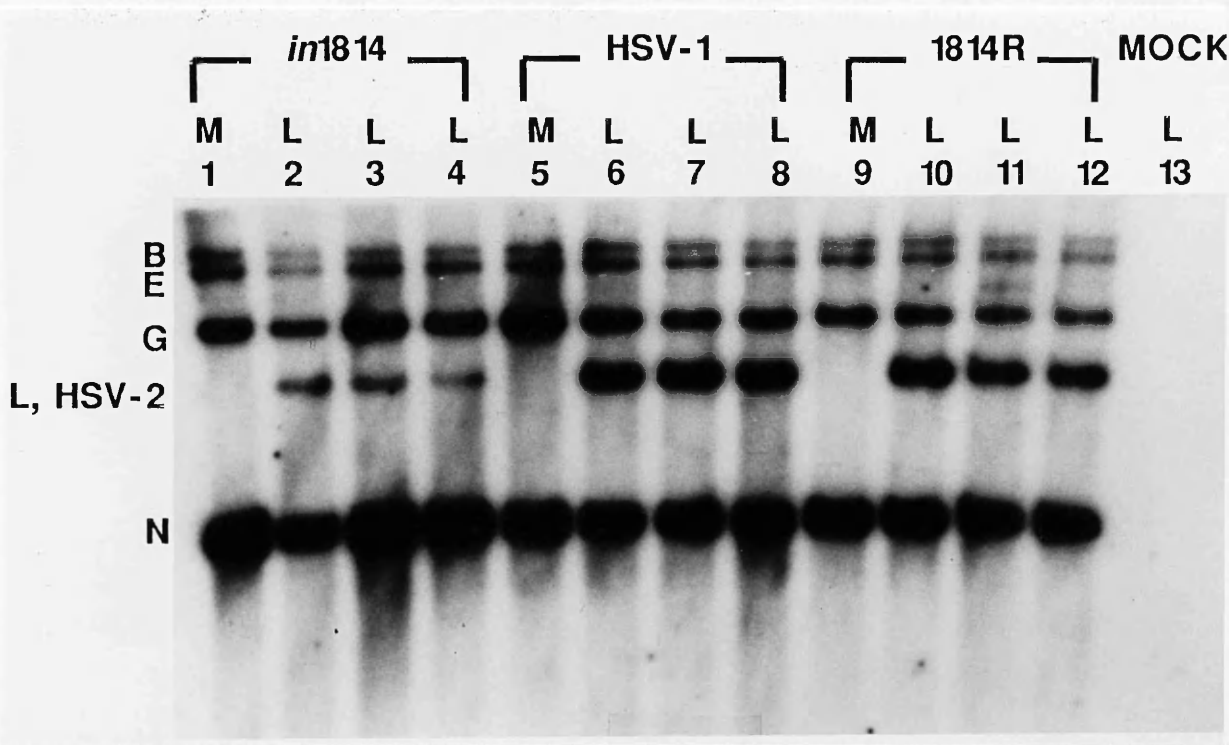
4.4.4.3. Reactivation of In1814 Requires Vmw110.

Reactivation of virus in the HSV-2 latency system is strictly dependent on Vmw110 supplied by a superinfecting virus (Russell et al., 1987; Harris et al., 1989). Further characterization of the in1814 latent infection in HFL cells was directed at determining whether a comparable requirement for Vmw110 was needed for the reactivation process.

HFL cells were mock infected or infected with 0.1 particles/cell of in1814 and incubated at 42°C for 6 days followed by 37°C for 4 days. Alternatively, latency was established at 37°C with 0.1 particles/cell in1814 in the presence of 50 μ g/ml Ara-C. Superinfection of monolayers was carried out, after removal of inhibitor where relevant, with 0.01-0.1 pfu/cell of dl1403 or wt HSV-1. Monolayers were harvested when cpe was evident, and progeny virus was used to reinfect a BHK cell monolayer. BamHI cleaved cytoplasmic DNA extracts were subjected to Southern blot hybridization

**Figure 4.12. REACTIVATION OF HSV-2 FROM LATENTLY INFECTED
CULTURES BY SUPERINFECTION WITH In1814.**

HFL cells were mock infected (M) or infected with 0.003 pfu/cell of HSV-2 (L) and incubated at 42°C for 6 days followed by 37°C for 4 days. Cultures were superinfected with in1814 (lanes 1 to 4), wt HSV-1 (lanes 5 to 8), 1814R (lanes 9 to 12) or mock superinfected (lane 13). Cytoplasmic DNA was extracted, cleaved with HindIII and hybridized to a labelled HSV-2 HindIII L fragment. The multiplicity of infection was 0.1 pfu/cell (lanes 2, 6 and 10), 0.001 pfu/cell (lanes 3, 7 and 11) and 0.001 pfu/cell (lanes 4, 8 and 12). Lanes 1, 5 and 9 contain samples infected with 0.01 pfu/cell.



analysis. Digestion of in1814 DNA with BamHI produces two novel BamHI fragments from BamHI f because of the presence of an additional site generated by BamHI linker insertion. Therefore, detection of two BamHI bands of about 5kbp and 3kbp against an 8kbp HSV-1 or dl1403 band is diagnostic of reactivated in1814. The Southern hybridization data (Figure 4.13.) show that superinfection with wt HSV-1, but not dl1403 or mock superinfection, induced the reactivation of latent in1814 after latency was established at 42°C or 37°C.

4.4.4.4. Discussion.

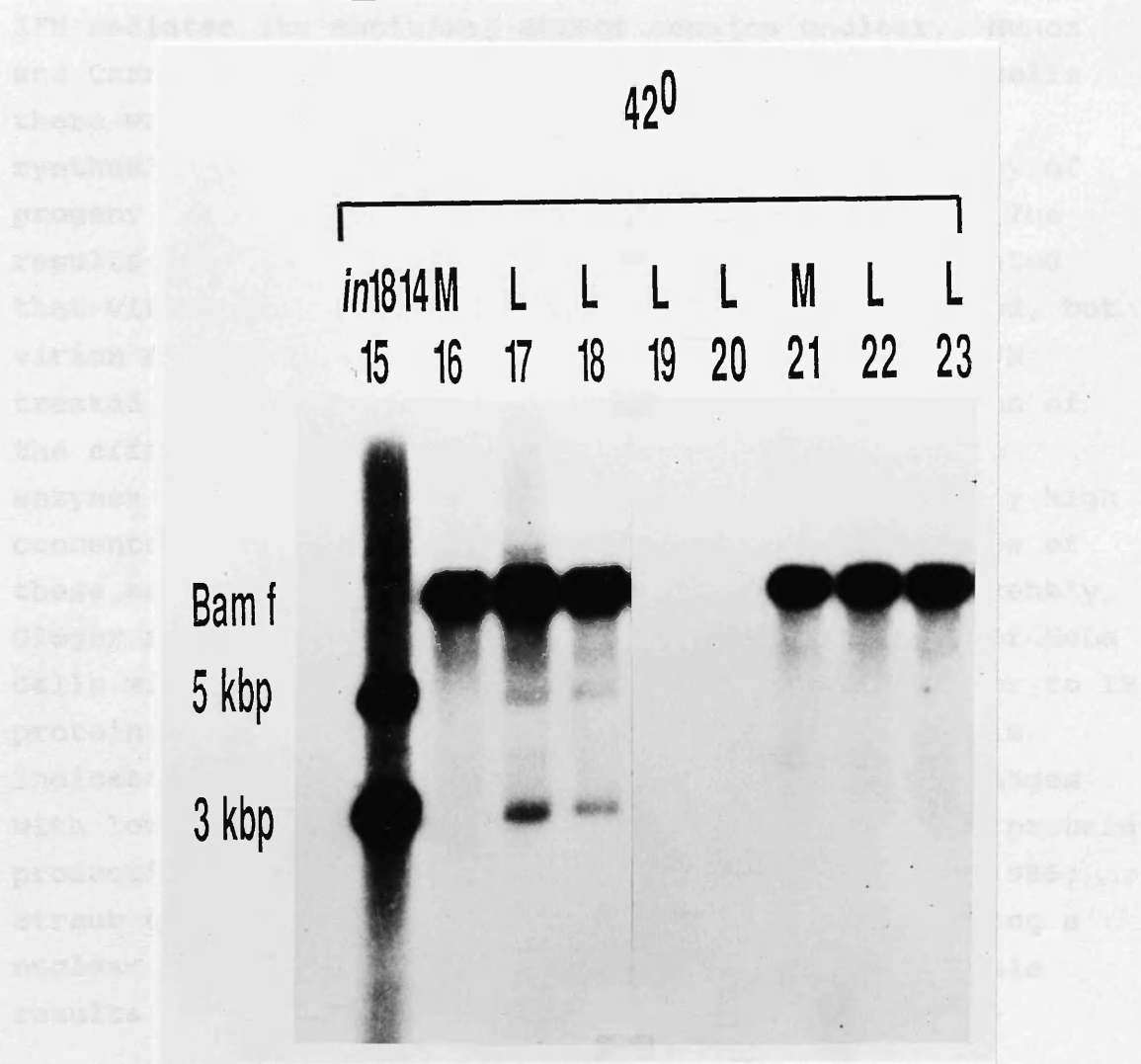
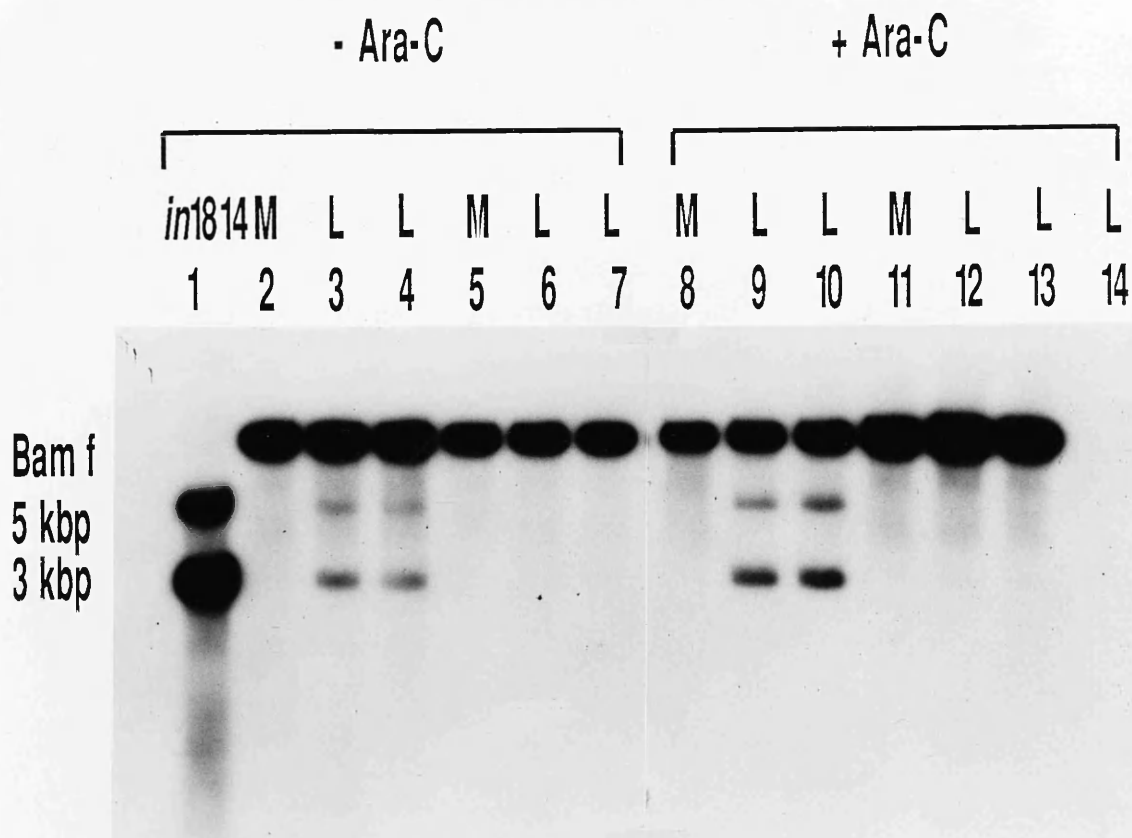
Recent experiments have demonstrated that in the mouse eye model for latency in1814 establishes a latent infection without detectable replication in the cornea or trigeminal ganglia and reactivates at a rate comparable to wt HSV-1 or 1814R. This implies that Vmw65 and viral replication are not essential for establishment of and reactivation from the latent state in vivo (Steiner et al., 1990). Furthermore, the ability of recombinant adenoviruses, expressing Vmw110 in the absence of other HSV proteins, to reactivate latent virus implies by default that Vmw65 is not a prerequisite for reactivation in vitro. The results described in this section demonstrate that, as expected, Vmw65 is not required for reactivation in vitro (Figure 4.12.).

Moreover, in analogy with the HSV-2 system, only a functional Vmw110 supplied by the superinfecting virus is necessary for reactivation of in1814 in either the 42°C or 37°C HSV-1 latency model system.

These results confirm the data derived from the time course experiment (Section 4.4.1.3.) in which Vmw65 failed to stimulate the latent viral genome whereas expression of Vmw110 by the superinfecting virus was essential for reactivation at 42°C or 37°C.

**Figure 4.13. REACTIVATION OF LATENT In1814 REQUIRES THE
POLYPEPTIDE Vmw110.**

HFL cell cultures were mock infected (lanes labelled M) or infected with 0.1 particles/cell in1814 (lanes labelled L). Latency was established in the absence of Ara-C at 37°C (lanes 2 to 7), at 37°C in the presence of 50µg/ml Ara-C (lanes 8 to 14) or at 42°C for 6 days followed by temperature downshift to 37°C (lanes 16 to 23). After incubation at 37°C for 4 days, and removal of inhibitor where appropriate, monolayers were mock superinfected (lanes 14, 19 and 20), superinfected with 0.1 pfu/cell wt HSV-1 (lanes 2-4, 8-10 and 16-18) or 0.1 pfu/cell dl1403 (lanes 5-7, 11-13 and 21-23). Cytoplasmic DNA was digested with BamHI. Lanes 1 and 15 contain 1µg BamHI cleaved in1814 DNA. The Southern blot was probed with a 1.6kb BamHI fragment derived from pMC17 (Ace et al., 1988).



4.5. The Effect of Interferon on Mutant In1814.

4.5.1. Introduction.

The antiviral effect of IFN in susceptible cells is reliant upon the de novo or increased synthesis of two double stranded RNA-dependent cellular enzymes. These are a 2',5'-oligoadenylate synthetase and a protein kinase which inactivates an initiation factor of eukaryotic protein synthesis, EIF2 (Baglioni, 1979). The synthetase activity is induced in murine trigeminal ganglia acutely infected with HSV-1 (Sokowa et al., 1980), although it has been suggested that some DNA viruses, including HSV, may have developed means to overcome these cellular defense mechanisms (Cayley et al., 1984).

IFN inhibits different viruses at various stages of virus replication. The inhibition of HSV growth by IFN has been demonstrated in several studies but the level at which IFN mediates its antiviral effect remains unclear. Munoz and Carrasco (1984) reported that in IFN pretreated cells there was no significant reduction in HSV-1 protein synthesis or virion assembly, although the infectivity of progeny virus produced was substantially decreased. The results presented by Chatterjee et al., (1985) indicated that virus specific protein synthesis was not affected, but virion morphogenesis and release of particles from IFN treated cells was prevented. In contrast, examination of the effects of IFN on the HSV-1 TK and DNA polymerase enzymes in mouse L-cells has shown that, at relatively high concentrations, IFN suppressed the specific activities of these early proteins (Panet and Falk, 1983). Subsequently, Gloger and Panet (1984) demonstrated that treatment of HeLa cells with IFN inhibited HSV-1 replication at or prior to IE protein synthesis. Similarly, an independent analysis indicated that pretreatment of mouse or human macrophages with low doses of IFN efficiently inhibited virus IE protein production (Domke et al., 1985; Domke-Opitz et al., 1986; Straub et al., 1986). Extension of both studies, using a nuclear runoff transcription assay, produced compatible results which indicated that the major target of IFN-

mediated inhibition is HSV-1 IE gene transcription (Mittnacht et al., 1988; Oberman and Panet, 1988,1989). These later reports have led to the attractive hypothesis that the IFN induced reduction of IE mRNA synthesis is a consequence of an inhibitory effect involving Vmw65 or the interaction of Vmw65 with cellular proteins. However, it is possible that more than one mechanism may be involved for HSV-1 or that different mechanisms can predominate in different cell types.

Interest in IFN and its application to latency in tissue culture arose from several observations. It has been demonstrated that IFN inhibits the growth of HSV in cell culture. In addition, treatment of HEL cells with IFN- α results in a significant increase in the proportion of cells in which a latent infection can be established (Wigdahl et al., 1982). Also, the availability of a Vmw65 mutant provided an opportunity to test the recent suggestion that inhibition of HSV-1 replication by IFN involves a specific effect on this polypeptide (De Stasio and Taylor, 1990).

The objective of the experiments described in this section was to determine if the effect of human lymphoblastoid IFN- α (Hu IFN- α Ly) acted additively upon in1814, which is already impaired for growth and gene expression on HFL cells, such that a high multiplicity latent infection could be established, thereby improving the sensitivity of the system.

4.5.2. Inhibition of Virus Plaque Formation on IFN-Treated HFL Cells.

Confluent monolayers of HFL cells were treated with 10^2 , 10^3 IU/ml of Hu IFN- α Ly for 18hr or were untreated. The medium was removed, monolayers were washed twice with medium without Hu IFN- α Ly, and then the plates were infected with 3-fold serial dilutions of in1814 or 1814R. After 1hr the virus inoculum was removed and the plates were overlaid with EHu2 supplemented with the appropriate amounts of Hu IFN- α Ly. 2-3 days later the plates were stained with giemsa and plaque numbers determined.

The results presented in Table 4.7. indicate that pretreatment of HFL cells with Hu IFN- α Ly is capable of reducing the titre of in1814 at least as efficiently as the rescued virus, and that the degree of inhibition is dependent on the concentration of Hu IFN- α Ly employed. The maximum reduction in titre, approximately 20-fold, was observed when HFL cells were pretreated with 10^3 IU/ml of Hu IFN- α Ly in parallel titrations of in1814 and 1814R.

4.5.3. Uptake of Viral DNA to the Nucleus in IFN-Treated HFL Cells.

The effect of Hu IFN- α Ly on the efficiency of DNA migration to the cell nucleus was determined. HFL cell growth medium was replaced with 5ml medium containing no Hu IFN- α Ly or 10^2 , 10^3 IU/ml Hu IFN- α Ly and incubation was continued overnight at 37°C. Prior to infection, the medium was aspirated and the monolayers washed twice to remove residual Hu IFN- α Ly. Monolayers were infected in the presence of 25 μ g/ml cycloheximide with 300 particles of in1814 or 1814R per cell. Nuclei were prepared 5hr post infection and BamH1 digested nuclear DNA was analyzed by Southern blot hybridization (Figure 4.14.). No significant differences were detected in the levels of HSV DNA showing that Hu IFN- α Ly does not prevent the transport of viral DNA to the nucleus, nor does it reduce the amounts of DNA reaching the nucleus.

4.5.4. Discussion.

The results described here imply that IFN mediated inhibition of HSV-1 growth is not at the level of transactivation by Vmw65, since in1814 is defective for IEC formation and thereby reduced for IE gene expression, and the mutant was not resistant to the antiviral effects of Hu IFN- α Ly being inhibited for growth to the same extent as wt virus. A complication of this experiment is that it may focus on the subpopulation of cells capable of supporting in1814 plaque formation. If a homologue of Vmw65 existed in these cells then the effect of Hu IFN- α Ly may be to block

Table 4.7. THE EFFECT OF PRETREATMENT OF HFL CELL MONOLAYERS WITH HU IFN- α LY ON THE TITRE OF In1814 AND 1814R.

VIRUS	No IFN	TREATMENT ^a	
		10 ² IU/ml	10 ³ IU/ml
1814R	1.0 x 10 ^{9b}	2.0 x 10 ⁸	5.8 x 10 ⁷
		(5) ^c	(17)
<u>in1814</u>	2.7 x 10 ⁶	4.4 x 10 ⁵	1.3 x 10 ⁵
		(6)	(21)

a Monolayers of HFL cells were treated with medium which lacked IFN or with medium containing Hu IFN- α Ly as indicated for 18hr prior to infection in duplicate with 3-fold serial dilutions of in1814 of 1814R.

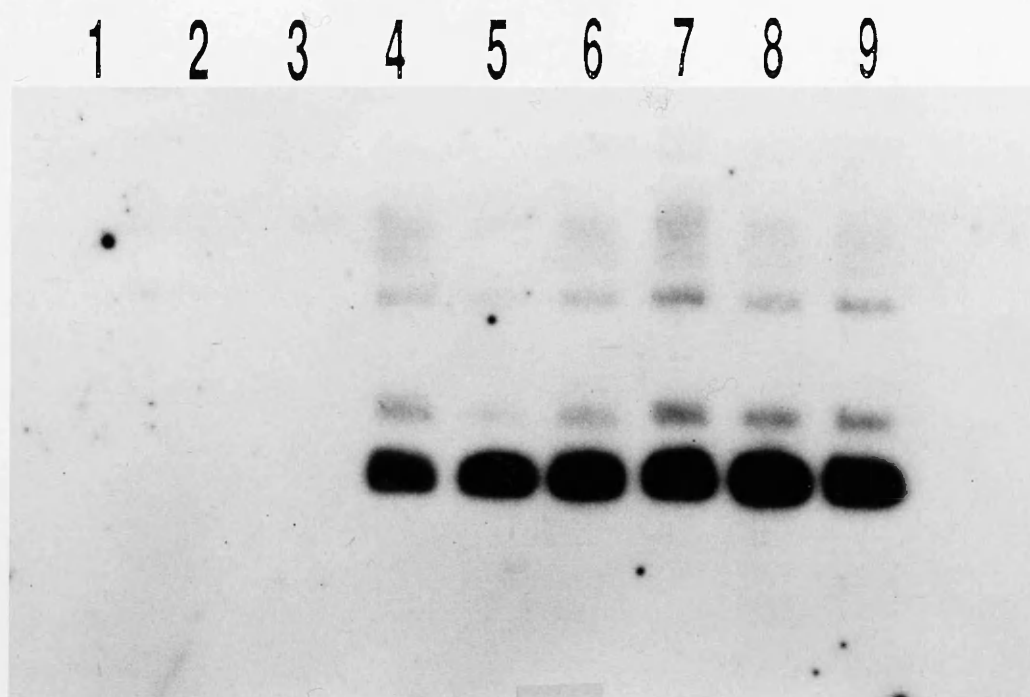
b Titre in terms of pfu/ml.

c Figures in parentheses are the fold reduction in titre in comparison to the titre in the absence of Hu IFN- α Ly.

Figure 4.14 EFFECT OF Hu IFN- α Ly ON MIGRATION OF VIRAL DNA TO THE CELL NUCLEUS.

Monolayers were treated overnight with either 10^2 IU/ml or 10^3 IU/ml Hu IFN- α Ly or were untreated. DNA was isolated from the nuclei of mock infected HFL cells (lanes 1 to 3) or from those infected in the presence of 25 μ g/ml cycloheximide with 300 particles of in1814 or 1814R per cell (lanes 4 to 6 and lanes 7 to 9 respectively). BamHI cleaved DNA was separated on an agarose gel, transferred to nylon membrane and probed with pTK1. The position of BamHI p is indicated.

Bam p



the action of such a homologue. However, co-infection of HFL cells with UV irradiated virus and in1814 increased the titre of in1814 essentially to wt levels in normal cells and in Hu IFN- α Ly treated cells suggesting that transactivation is not affected by IFN treatment (C.M. Preston, unpublished observation). The Southern blot data (Figure 4.14.) demonstrate that virus migration of viral DNA to the cell nucleus is not affected by Hu IFN- α Ly treatment. Likewise, this is implied from in vitro latency experiments (S. Jamieson, unpublished observations) which show that the long term potential gene expression is not affected.

It is speculated that the inhibition of in1814/1814R replication by Hu IFN- α Ly involves an effect on IE transcription in analogy with other published data (Mitnacht et al., 1988; Oberman and Panet, 1988, 1989). However, this possibility has not ^{been} investigated and alternatively, the inhibitory mechanism of IFN could be effective at late stages of virus replication (Munoz and Carrasco, 1984; Chatterjee et al., 1985). The results presented in this section cannot explain why IFN specifically reduces IE transcription. In contrast to recent evidence (De Stasio and Taylor, 1990), it is clear that it is not achieved at the level of Vmw65.

Wigdahl et al. (1984a) detected linear genomes in their in vitro latency system which is reliant on the use of IFN- α in combination with BVDU to repress virus replication. The presence of these linear genomes may have arisen as a consequence of a block to transport of viral DNA to the nucleus mediated by IFN- α treatment. The results shown in Figure 4.14. predict that this was not the case, and in the context of this experiment the observation by Wigdahl et al. (1984a) remains unexplained.

5. FINAL DISCUSSION.

To date no specific viral gene products have been identified which are associated with the establishment of latent infections without affecting replication. However, it has been hypothesised that the decision between a latent infection or progression to lytic phase gene expression may be determined by the presence or absence of the tegument protein, Vmw65, in the nucleus of the sensory ganglia. This balance may be altered by a variety of factors including the failure of sufficient Vmw65 to be transported to the neuronal nucleus, perhaps as a consequence of a low multiplicity infection, lack of necessary cellular transcription factors, like OTF-1, for IE gene expression in certain neurones (Roizman and Sears, 1987), or functional alteration of Vmw65.

Use of a Vmw65 mutant, in1814, in latency studies has demonstrated that a functional polypeptide is not required for establishment or/^{explant}reactivation of latency in the mouse eye model (Steiner et al., 1990). Moreover, in1814 replication at the site of inoculation was not detected (Steiner et al., 1990). These findings imply that Vmw65 is not required for establishment, nor is virus replication an essential prerequisite for establishment of latency, and that in1814 is predisposed to latent infection in vivo. Likewise, during latency in tissue culture, in1814 behaves in a similar mannner to the observed in vivo situation in terms of its ability to establish latency, in particular the capacity for high multiplicity latent infections at 42°C or at the physiological temperature 37°C (Section 4.4.1.). The predisposition of in1814 to latency argues for a defect in IE gene expression mediated at the level of Vmw65 being the actual pathway through which latency occurs. Furthermore, results presented in Section 4.4.1.2. suggest that incubation of cells at the supraoptimal temperature of 42°C renders the HSV genome incapable of synthesising adequate levels of IE gene products presumably by interfering with Vmw65 function.

It has been shown that HSV TK mutants do not replicate in murine sensory ganglia but retain the ability to

establish latency in these tissues (Coen et al., 1989; Efstathiou et al., 1989; Leist et al., 1989; Tenser et al., 1989). In this regard, these mutant viruses have a phenotype similar to in1814. The pattern of viral gene expression in murine trigeminal ganglia of defined TK⁻ viruses has been examined using in situ hybridization with probes for all classes of HSV gene, including LAT (Kosz-Vnenchak et al., 1990). During latent infections of trigeminal ganglia TK⁻ mutant strains expressed nuclear LAT RNA but lytic gene expression, especially IE gene expression, was at very low or undetectable levels (Kosz-Vnenchak et al., 1990). It is not clear why a defective gene encoding TK, a prototypic early gene should result in decreased amounts of IE gene products. However, the results are consistent with observations employing in1814 which is also defective for IE gene expression but can establish a latent infection (Steiner et al., 1990; Section 4.4.1.). Therefore, the implication of these data is that preferential establishment of latency in the absence of IE gene expression is not unique to a Vmw65 mutant.

In an attempt to clarify the role of Vmw65 during establishment of latency, two recombinant viruses have been constructed in which Vmw65 transcription was controlled by the mouse metallothioneine promoter (R603) or a stop codon was inserted into the normal Vmw65 gene (R604). Normal mice or transgenic mice (which constitutively expressed Vmw65) were infected with viruses R603 and R604, and tested for the establishment of latent infections. Both recombinants established latency in normal mice with equivalent frequency and likewise, the expression of Vmw65 in transgenic mice, induced by heavy metals had no effect on the capacity of these viruses to establish a latent infection. Therefore, these data from Roizman's group suggest that the postulated lack of Vmw65 and the consequential reduction in IE gene expression is not operational during the establishment of latent infections in mice (Roizman and Spector, 1991).

Recent evidence has suggested the presence of a repressor activity specifically in cells of neuronal origin (C1300 cells). The presence of a trans-acting repressor molecule was implied from several lines of experimental

evidence including: i) co-transfection of an IE construct with another vector containing a single copy of the TAA^TGARAT sequence element increased expression of a heterologous gene, and ii) permissivity for HSV-1 infection in C1300 cells increased following prior transfection of a plasmid containing the TAATGARAT element presumably by sequestration of the neuronal repressor by excess TAATGARAT motifs (Kemp et al., 1990). Furthermore, the existence of a putative repressor protein specifically in C1300 cell nuclei was demonstrated by gel retardation assay using an overlapping consensus octamer/TAATGARAT oligonucleotide probe (Kemp et al., 1990). Therefore, these results imply that a failure of viral gene transcription due to the presence of an IE gene inhibitor in neurones may play a role in the establishment of latent infections.

In summary, a model for establishment of latency in a situation where IE gene expression is reduced or absent, due to an aberrant Vmw65 molecule as a consequence of one or a combination of the situations detailed above, fits the data presented in this thesis and elsewhere (Steiner et al., 1990). However, it also compares with observations using TK⁻ mutants (Kosz-Vnenchak et al., 1990). Therefore, a virus may favour a latent infection under more diverse circumstances, for example, low multiplicity of infection or because of any non-specific defect that interferes with IE gene expression. Indeed, certain HSV IE genes possess the ability to inhibit transactivation of target genes in transient expression systems (O'Hare and Hayward, 1985b; Su and Knipe, 1989). It is possible that these proteins may have a modulatory effect on the expression of viral genes facilitating a latent virus-cell interaction.

Models that rely on a simple loss of Vmw65 over the distance between the nerve endings and the neuronal nucleus or an IE inhibitory factor limited to cells of the nervous system fail to account for the evidence that HSV can exist in a latent form at the periphery in vivo in cells other than neuronal cells (Clements and Subak-Sharpe, 1988; Al-Saadi et al., 1988; Clements and Jamieson, 1989) and in vitro in non-neuronal cells (Russell and Preston, 1986; this thesis).

In vivo, the presence of the latent HSV genome has been detected in multiple copies per neuronal cell (Puga et al., 1978; Cabrera et al., 1980; Rock and Fraser, 1983,1985; Efsthathiou et al., 1986). On the basis of this evidence, it has been suggested that the balance between maintenance of the latent state and viral gene expression leading to lytic replication may depend on the copy number of viral genomes per latently infected cell. Thus, any given stimulus may cause an indirect amplification of viral genomes such that eventually a tolerated level of genomes is exceeded and reactivation is induced. Such a model for HSV latency is not inconsistent with results described in Section 4.4.2. in which maximal input of in1814 genomes does not result in an incremental increase in levels of latent DNA compared with wt HSV-2 infections at a relatively low moi (Preston and Russell, 1991). Other supporting evidence suggests that there is a correlation between initial infection at high multiplicity and the level of spontaneous reactivation from latency in the in vitro model system (Russell, 1989).

The absolute copy number of EBV genomes varies between different latently infected cell lines but is generally stable over time within a given cell line. Several viral proteins are expressed in cell lines harbouring latent EBV. Of these, only EBNA-1 is expressed in virtually all cells examined which retain latent EBV genomes. EBNA-1 interacts with a latent phase origin of replication (oriP) which is distinct from the origin used during lytic infection (Lupton and Levine, 1985; Yates et al., 1985) and therefore, oriP and EBNA-1 are functionally important for the controlled replication and maintenance of latent EBV episomes in proliferating cells. OriP is composed of a 65bp region of dyad symmetry, which functions as the actual origin of replication (Wysokenski and Yates, 1989), and a family of tandem, imperfect repeats which act as a transcriptional enhancer of heterologous genes that is strictly dependent on the presence of EBNA-1 (Reisman et al., 1985; Reisman and Sudgen, 1986). Rawlins et al. (1985) have demonstrated that a fusion protein representing the C-terminus portion of EBNA-1 binds specifically to the repeat sequences in vivo. Recently, the enhancer region within oriP has been shown to

increase transcription from an EBV promoter region, in combination with EBNA-1 supplied in trans, that is responsible for the expression of some of the latent phase proteins, including EBNA-1 (Sugden and Warren, 1989). Thus, EBNA-1 is important for the synthesis of latent viral DNA and can potentially affect transcription from the latent EBV genome.

Two pieces of evidence predict that the control of genome copy number analogous to that observed in B-lymphocytes latently infected with EBV is not applicable to HSV latent infections. First, the EBV genome is replicated by the cellular DNA polymerase, in the context of the viral requirements discussed above, concomitantly with the replication of the dividing B-lymphocyte (Adams, 1987). Clearly, since neuronal cells invariably do not divide, the mechanisms involved in genome amplification must differ at this level in these divergent cell types. Moreover, the finding that HSV-1 LAT or mLAT mutants are capable of establishment and maintenance of latency in the absence of viral gene expression (Javier et al., 1988; Leib et al., 1989a; Steiner et al., 1989; Sedarati et al., 1989; Mitchell et al., 1990b) implies that a similar EBNA-1 function is not required during HSV-1 latent infections.

Alternatively, herpesvirus latency may be controlled by mechanisms that involve the modification of the latent genome. Elucidation of the role of DNA methylation has typically involved the use of isochizomers which share the same recognition site but are differentially sensitive to methylation at CG dinucleotides, or the use of DNA hypomethylating agents. At least a proportion of CpG residues are methylated during non-productive EBV and HSV infections (Desrosiers et al., 1979; Szyf et al., 1985.). The demethylating agent 5-azacytidine has been shown to activate virus production in lymphoid cells containing latent EBV (Ben-Sasson and Klein, 1981). This agent and other demethylating agents increase the reactivation efficiency of HSV from its latent state in vivo (Whitby et al., 1987; Bernstein and Kappes, 1988; Stephanopoulos et al., 1988) which suggests that, like other herpesviruses, methylation of latent HSV DNA and subsequent hypomethylation

may be involved in the maintenance of latency and the switch to lytic gene expression. In contrast, there is conflicting evidence which indicates that HSV-1 DNA is not heavily methylated in vivo (Dressler et al., 1987) and certainly, the usual demethylating agents are not effective in stimulating reactivation of latent virus in tissue culture (Russell, 1989; Section 4.4.3.1.). Therefore, the cumulative evidence may point to a limited methylation of the HSV genome, perhaps including the IE promoter regions. On the other hand, since most demethylating agents can affect a broad spectrum of cellular processes, analysis of the alterations in cellular metabolism may indicate interesting events that lead to the reactivation of a latent genome that is not significantly methylated.

In the mouse eye model system, the majority of latent HSV-1 DNA is arranged in a complex structure that resembles cellular chromatin, whilst during the acute infection most viral DNA is not associated with nucleosomes (Deshmane and Fraser, 1989). Although control of viral gene expression may be determined crudely at the level of chromatin formation, other factors must be important as the region of the genome encoding LAT is associated with nucleosomes (Deshmane and Fraser, 1989), and yet transcription is not inactivated.

Recent studies have defined an area of the HSV genome that is active during latency in latently infected ganglionic nuclei of the natural host and experimental animals (Stevens et al., 1987,1988; Rock et al., 1987a; Spivack et al., 1988; Krause et al., 1988). These transcripts are termed LAT, their properties and biological significance are discussed in detail in Section 1.3.5.4. RNA with at least some of the properties of LAT have been demonstrated in rabbits latently infected with BHV-1 (Rock et al., 1987b) and PRV (Rock et al., 1988; Lokensgard et al., 1990; Priola et al., 1990). However, there is a fundamental disparity between the cellular localization of the PRV latency specific RNAs; in one case nuclear retention of RNA has been reported (Rock et al., 1988) whilst another group has detected cytoplasmic species (Priola et al.,

1990). In situ hybridization analysis of latently infected human trigeminal ganglia has indicated that the VZV latent phase transcription pattern differs from HSV-1 (Croen et al., 1988). Transcripts were mapped in the opposite polarity to some genes expressed during lytic infection of tissue culture cells and were apparently not restricted to regions of the genome encompassing the IE genes (Croen et al., 1988). Thus, there are obvious similarities and differences in the status of latent gene expression amongst alphaherpesviruses which may yet involve a common mechanism, perhaps in analogy with the HSV-1 situation wherein some property of the transcriptionally active region appears to have a role in facilitating reactivation from the latent state (Leib et al., 1989a; Steiner et al., 1989; Sedarati et al., 1989; Hill et al., 1990).

A possible role for the involvement of the HSV-1 LAT region in the control of latency has been postulated based on observations that LAT RNA is the only species abundantly transcribed during latency and that it is antisense to the mRNA of the IE protein Vmw110 (Stevens et al., 1987; Rock et al., 1987a; Figure 1.6.). Recent evidence suggests that this theory may be credible since LAT has the characteristics of a stable intron that can inhibit the transactivation function of Vmw110, at least in transient transfection assays (Farrell et al., 1991). This property is not without precedent as antisense RNA molecules with important regulatory function have been reported in Xenopus and differentiated murine erythroleukaemia cells (Kimelman and Kirschner, 1989; Khochbin and Lawrence, 1989). Interestingly, the specificity of LAT RNA inhibition is apparently limited to Vmw110 which is important for reactivation of HSV from its latent state in vitro (Russell et al., 1987; Russell, 1989; Section 4.2.). In this context it is important to note that Vmw110 apparently plays a role in the synthesis of infectious virus following transfection of viral DNA, a process that may mimic events occurring during reactivation (Cai and Schaffer, 1989). Therefore, a negative effect of LAT RNA on production of Vmw110 mRNA or protein might ensure the maintenance of latency. However, in vivo studies have demonstrated that there is no

requirement for Vmw110 in the reactivation process (Leib et al., 1989b; Clements and Stow, 1989).

Preliminary evidence has suggested the existence of a protein product derived from the region transcribed during HSV latency (Doerig et al., 1990, abstracts of the 15th International Herpesvirus Workshop, Washington DC, USA). The promoter region controlling HSV latent phase gene expression contains a functional CRE that is important for the cAMP-mediated acceleration of reactivation by LAT⁺ viruses but not a LAT⁻ virus that lacks the CRE and other sequences specifying LAT (Leib et al., 1991). In the context of this recent result, it is plausible that reactivation stimuli act indirectly on such response elements and induce the synthesis of a latency specific polypeptide that is responsible for the disruption of the latent infection.

In lymphoid cells latently infected with EBV, several chemically unrelated agents induce lytic expression from the viral genome, such as butyrate, calcium ionophores, tumour promoters, anti-immunoglobulins and a factor present in serum. Some of these agents are thought to act by stimulation of the cellular protein kinase C. The mechanism by which modification of intracellular protein kinase C concentrations induces expression from the EBV genome has not been elucidated, but it probably involves the induction of a protein transcribed from the BamHI Z leftward ORF (BZLF1). BZLF1 is a sequence specific DNA-binding protein with homology to the product of the c-fos gene (Farrell et al., 1989) that has the ability to transactivate some cellular and viral promoters (Rooney et al., 1989; Flemington and Speck, 1990). Transfection of a BZLF1 construct is sufficient to induce the EBV productive cycle (Countryman and Miller, 1985; Takada et al., 1986; Grogan et al., 1987). Furthermore, the primary transcript for the gene encoding EBNA-1, which is expressed continuously in latently infected lymphocytes, extends across the BZLF1 gene in an antisense direction (Baichwal and Sugden, 1988).

In summary, an external stimulus such as exposure to UV irradiation, fever, or stress may trigger an alteration in the expression of neuronal genes, perhaps resulting in the

physiological induction of a cell protein which functions in a similar manner to Vmw110 or BZLF1. This may explain the disparity between the necessity for Vmw110 in the reactivation process in tissue culture latency systems, and conversely, the absence of this requirement in animal model systems. The putative latency specific polypeptide, presumably synthesised from polyadenylated mLAT, may be produced downstream in the reactivation pathway as a consequence of the activities of a cellular protein(s).

The experimental data presented in this thesis extend the characterization of in vitro latency in HFL cells. Initial experiments using wt HSV-2 demonstrated that a stable latent infection is established by prolonged incubation of infected HFL cells at the supraoptimal temperature, 42°C, a process that requires very little or no viral gene expression (Russell and Preston, 1986; Russell et al., 1987; Russell, 1989). Use of the Vmw65 mutant in1814, which is blocked at a very early stage of infection prior to expression of IE gene products, permits the establishment of a high multiplicity latent infection at 42°C, or directly at the physiological temperature with the caveat that an inhibitor of viral DNA synthesis is incorporated in the growth medium to repress the replication of a background of non-latent particles. Therefore, Vmw65 is not needed for the establishment of latency in vitro. Indeed, its absence predisposes the virus to latent infections. Mutant in1814 has facilitated the examination of the molecular mechanisms underlying HSV-1 latency in vitro. In particular, analysis of the physical organization of the latent genome has indicated that the HSV-1 genome is maintained in a form that is predominantly non-linear. This provides confirmation of earlier evidence that during HSV-2 latency in tissue culture cells, the viral genome is converted to an "endless" configuration at 42°C (Preston and Russell, 1991). Concomitantly with the conversion of in1814 DNA to a non-linear molecule, the IE promoters undergo rapid modification such that they are refractory to Vmw65 but retain the capacity to respond to Vmw110 supplied in trans. These alterations probably reflect the packaging of the viral

genome into a conformation that resembles chromatin and that like HSV-2, reactivation of in1814 in the normal 42°C system or at the permissive temperature is dependent on the presence of functional Vmw110. Viral gene expression is suppressed in HFL cells latently infected with in1814 (Section 4.4.3.). Although the absence of LAT RNA is an important difference from the observed in vivo situation, it is apparent that there is no requirement for LAT in the processes governing establishment and maintenance. Furthermore, the role of LAT and mLAT in reactivation has not been precisely defined.

As a consequence of the results presented in this thesis and by others, which are described above, it is clear that in many respects the virus-cell interaction observed in vitro closely resembles in vivo phenomena and as such will continue to contribute to the understanding of the molecular biology of HSV latency. In this regard, the most important areas of the virus-cell interaction in vitro that warrant further investigation encompass the following areas: i) analysis of the mechanism by which Vmw110 stimulates reactivation of latent virus, ii) comprehensive investigation of the physical organization of the latent genome and, iii) examination of viral gene expression, and its control during latency.

A more detailed analysis of the functional domains of Vmw110 may highlight other additional areas, distinct from the cysteine-rich motif (Harris et al., 1989; Section 4.2.2.), that are also important for reactivation. This type of analysis is amenable, as a large panel of mutant viruses deleted for small regions of Vmw110 are available (Everett, 1989), and would facilitate a comparison with independent observations in which adenoviruses expressing deleted forms of Vmw110 were assayed for the potential to recover latent HSV-2 in the 42°C latency system (Zhu et al., 1990).

A contention between in vitro and in vivo latency studies is the differential requirement for Vmw110, which probably reflects the differences in reactivation in HFL cells induced by superinfection and spontaneous reactivation

in vivo, and thereby the involvement of the neurone in the events that govern reactivation. Moreover, in animal systems, host species, virus strain and the initial moi are determinants of the necessity for a functional Vmw110 (Clements and Stow, 1989; Gordon et al., 1990b). The identification and characterization of the putative cellular protein homologous to Vmw110 is a very ambitious undertaking. An interesting starting point may be 2.5BHK cells, which are documented to increase the plaquing ability of Vmw110 mutants (Everett, 1989) and therefore presumably synthesize a complementing polypeptide. Furthermore, HMBA and other demethylating agents are reported to increase the efficiency of reactivation at explant (Whitby et al., 1987; Bernstein and Kappes, 1988; Stephanopoulos et al., 1988) in a manner that may not entail hypomethylation of latent DNA. Thus, examination of the mode of action of such agents may provide information on the nature of the proteins involved in the events that lead to reactivation.

Having defined that the latent in1814 genome lacks the terminal restriction endonuclease fragments, it is important that other features of latent DNA are examined. For example, is HSV-1 DNA in a circular form and associated with nucleosomes during latency in vitro, in analogy with observations in the mouse eye model system (Rock and Fraser, 1985; Mellerick and Fraser, 1987; Deshmane and Fraser, 1989)? Although the methylation status of latent HSV DNA has been investigated using demethylating agents (Russell, 1989; Section 4.3.1.), it will also be important to complement these studies with an analysis of the restriction profile of latent DNA using enzymes, such as SmaI and XmaI, that are differentially sensitive to methylation at the target sequence.

The absence of detectable LAT RNA in vitro (Section 4.4.2.3.) dictates that the further analysis of this RNA must involve a modification of the existing latency system. Since a neuronal specific element is present in the upstream region of the basic LAT promoter (Batchelor and O'Hare, 1990; Zwaagstra et al., 1990), this may be achieved by use

of primary neuronal cultures or established cell lines of neuronal origin, such as C1300 cells, which are more accessible. The other option involves the construction of a mutant virus, based on in1814, in which the region that confers high level expression of LAT in neurones is specifically removed and replaced with a strong enhancer to drive LAT gene expression in HFL cells.

Concerning the suggestion that mild heat shock of HFL cells and mutations in Vmw65 interfere with the same process (Section 4.4.1.2.), this possibility could be investigated using gel retardation assays of nuclei extracted from uninfected and infected HFL cells incubated at 42°C.

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